U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Effects of dietary creatine supplementation in gilthead seabream (*Sparus aurata*) muscle growth.

Lourenço de Oliveira Coelho e Ramos Pinto

Dissertação de Mestrado Apresentada ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto – Ciências do Mar e Recursos Marinhos

Lourenço de Oliveira Coelho e Ramos Pinto

Effects of dietary creatine supplementation in gilthead seabream (*Sparus aurata*) muscle growth.

Dissertação de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos.

Submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

Orientador – Professora Doutora Luísa Maria Pinheiro Valente.

Categoria – Professor Associado com agregação

Afiliação – Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Co-orientador – Professor Doutor Pedro Miguel Leal Rodrigues.

Categoria – Professor Auxiliar.

Afiliação – Universidade do Algarve – Faculdade de Ciências e Tecnologia

Acknowledgements

The conclusion of this work had important contributions, without which this research would not have been possible. Firstly, I would like to thank Professor Luísa Valente and Professor Pedro Rodrigues for accepting me to enroll as a student in this research. Especially I wish to thank Professor Luísa Valente for her support and for everything she taught me throughout this research project, helping me to improve my research skills, always helping me to see the right path.

To Professor Filipe Castro and Dr. Isabel Cunha that shared their knowledge in the genetics area. Thank you, Professor Filipe, for the cooperation and availability of your lab to develop this work.

To the coordinator of the Masters in Marine Sciences and Marine Resources, Professor Eduardo Rocha, I thank the cooperation and the help along the Master degree.

Thanks, Graciliana Lopes for all your advice, knowledge and patience that helped me through the molecular biology work. Thanks, Vera for your help and guidance during the histology part of the work. And to everyone in the LANUCE team that one way or the other helped me.

Thank you, mother and sister, for all your unconditional support!

Finally, but not least, many thanks to my great friends that were always there when I needed.

Abstract

Creatine (Cr) is nonessential amino acid and it has an important role in the cell as an energy buffer. Cr has been used as supplementation for many years to increase muscle strength and lean body mass in healthy individuals and athletes. But so far information of the use of dietary creatine supplementation and subsequent effects in fish is extremely scarce. Fish consumption is increasing worldwide. Moreover farmed fish has been increasing in the last decades contributing to world's fish consumption as the amount of captured fish has stagnated since the 1990s.

The aim of this work was to clarify and determine whether dietary creatine supplementation during the juvenile life stage of the gilthead seabream (*Sparus aurata)* affects muscle growth. Fish juveniles were fed *ad libitum* for 69 days with diets containing three increasing creatine monohydrate levels (0%, 2%, 5% and 8%) The white skeletal muscle was sampled at the end of the trial to evaluate muscle growth dynamics (dorsal muscular area (DMA), fibres density, fibres diameter) and the expression of muscle growth related genes.

Overall muscle cellularity was not significantly affected by dietary creatine supplementation, but the DMA increased significantly in fish fed 5% and 8% creatine. A tendency for an increase in muscle fibre diameter was observed with increasing creatine supplementation levels. The expression of most genes involved in myogenesis (*MyoD*1 and 2, *Mrf4*, *mstn* and *mhc*) increased relatively to the control group, but *MyoD1* was the only gene that was significantly upregulated with a 5% creatine dietary level. The gene family of calpains, involved in protein regulation, namely *CAPN1* and *CAPN3* relative expression were significantly affected by the dietary treatments. The current study showed that the dietary supplementation of 2% creatine had no impact on muscle cellularity nor in the expression of muscle-growth related genes, but a higher level resulted in increase dorsal muscle fibre area associated with the expression of key genes.

Keywords: Creatine supplementation, gilthead seabream (*Sparus aurata*), muscle growth, genes.

Resumo

A creatina é um conhecido aminoácido não essencial que tem um papel fundamental na dinâmica celular como acumulador de energia. A suplementação de creatina tem vindo a ser grandemente utilizada há muitos anos com o objetivo de aumentar a força muscular e a massa corporal magra em atletas e indivíduos saudáveis. No entanto, a informação sobre a sua utilização e os seus efeitos subsequentes em peixes é extremamente escassa. Atualmente, o consumo de pescado têm vindo a aumentar em todo mundo. Além disso, o peixe proveniente de aquacultura tem vindo a aumentar nas ultimas décadas e a contribuir significativamente para o consumo mundial, uma vez que a quantidade de peixe capturado estagnou desde os anos 90.

O presente estudo tem como objetivo esclarecer e determinar os efeitos da creatina como suplemento nutricional sobre o crescimento muscular durante a fase juvenil da dourada (*Sparus aurata).* Assim, juvenis desta espécie foram alimentados *ad libitum* por 69 dias num circuito fechado, com dietas suplementadas com concentrações crescentes de creatina monohidratada (0%, 2%, 5% e 8%). No final da experiência, foi amostrado o músculo esquelético branco para avaliar a dinâmica de crescimentos do músculo dorsal (área muscular dorsal, diâmetro das fibras e densidade das fibras) e a expressão de genes relacionados com o crescimento muscular.

Em termos gerais, os parâmetros do crescimento muscular não foram significativamente afetados com a suplementação de creatina, apenas a área muscular dorsal aumentou significativamente com o maior valor observado no grupo suplementado com Cr 5% e 8%. Contudo, observou-se uma tendência para um aumento do diâmetro das fibras muscular com o aumento crescente da suplementação nutricional testada. O nível de expressão dos genes envolvidos na miogénese (*MyoD*1 e 2, *Mrf4*, *Mstn* e *mhc*) aumentou significativamente relativamente ao grupo controlo, no entanto apenas a expressão do gene *MyoD*1 alterou significativamente entre tratamentos, com um pico na suplementação de 5% de creatina. Relativamente aos genes da família das calpaínas envolvidos na regulação proteica, a expressão dos genes *CAPN1* e *CAPN3* foi significativamente afetada em resposta a suplementação com creatina. Este trabalho revela que a suplementação nutricional com creatina de 2% não teve impacto na celularidade muscular nem na expressão de genes de crescimento relacionados com o músculo, mas numa percentagem de suplementação mais elevada aumentou a área muscular dorsal associada à expressão de genes principais.

Palavras-chave: suplementação de creatina, dourada (*Sparus aurata*), crescimento muscular, genes.

Index

Abbreviations

ADP – adenosine diphosphate

ATP – adenosine triphosphate

ANOVA – one-way analysis of variance

CAPN – calpain

cDNA – complementary deoxyribonucleic acid

Cr – creatine

CK – creatine kinase

CSA – cross-sectional area

DMA – dorsal muscular area

DNA – deoxyribonucleic acid

DGI – daily growth index

GH – growth hormone

MHC – myosin heavy chain

MRFs – myogenic regulatory factors

mRNA – messenger ribonucleic acid

Mrf4 – myogenic regulatory factor 4

MPCs – myogenic progenitor cells

Myf5 – myogenic factor 5

Mstn – myostatin

PCr – phosphocreatine

qPCR – real-time polymerase chain reaction

Figures List

Tables List

1 Introduction

1.1 Species characteristic

The gilthead seabream (*Sparus aurata,* L.) is a marine teleost that belongs to the *Sparidae* family. It has an oval body, slightly deep and compressed, with a head regularly curved with small eyes. It has a silvery-grey body, and a large black spot at the origin of the lateral line that extends on the upper margin of the operculum, where a reddish area encloses it below (Fig. 1). It has a characteristic golden band between the eyes, bordered by two dark areas; the fork and tips of the caudal fin are edged with black (Basurco *et al.*, 2011). The gilthead seabream is commonly found in the Mediterranean Sea with the exception of the eastern and southeastern part of the Mediterranean where its presence is scarce. It is also present in the Eastern Atlantic but very rarely in the Black Sea. As a benthopelagic (demersal behaviour) and euryhaline species, gilthead seabream inhabits coastal environments of seagrass beds, rocky and sandy bottoms, and regularly enters briny waters where it can reach from 30 m to 150 m depth as an adult. It is a sedentary fish, either solitary or in small aggregations. It is mainly carnivorous (feeding on molluscs, particularly mussels which can be easily crushed, crustaceans and fish) and accessorily herbivorous (Basurco *et al.*, 2011).

In terms of its reproductive biology, the gilthead seabream is a protandrous hermaphrodite; the majority of individuals are functional males in the first two years (20-30 cm) and then turn into females (33-40 cm) (Sola *et al.*, 2007; Colloca & Cerasi, 2015). The spawning usually occurs from December to April, depending on social, environmental (photoperiod, water temperature) and genetic factors; but usually occurs when the water temperature ranges from 13 to 17 ºC (Basurco *et al.*, 2011). Due to the great importance of gilthead seabream for the marine aquaculture, there has been an increase of research exploring its physiology, nutrition, immune response, growth performance reproduction and genetics (Basurco *et al.*, 2011).

Fig.1. *Sparus aurata* (Linnaeus, 1758).

1.2 Current status of gilthead seabream production

The total gilthead seabream harvest in 2012 was 167.826 megatonnes (MT), with 95.2% (159.730 MT) of its production coming from aquaculture (Fig. 2) and the remaining 4.8% (8096 tonnes) coming from capture fisheries according to FAO (2014).

Most gilthead seabream production occurs in the Mediterranean, with Greece (49%) being the largest producer, followed by Turkey (15%), Spain (14%) and Italy (6%). In addition, significant production occurs in Cyprus, Croatia, Egypt, Morocco, France, Malta, Portugal and Tunisia (Colloca & Cerasi, 2015). This species is traditionally farmed in Mediterranean countries alongside with the European sea bass (*Dicentrarchus labrax,* Linnaeus 1758). Their production occurs usually by the same companies and frequently in the same farms, but the two species are generally cultured separately (Basurco *et al.*, 2011).

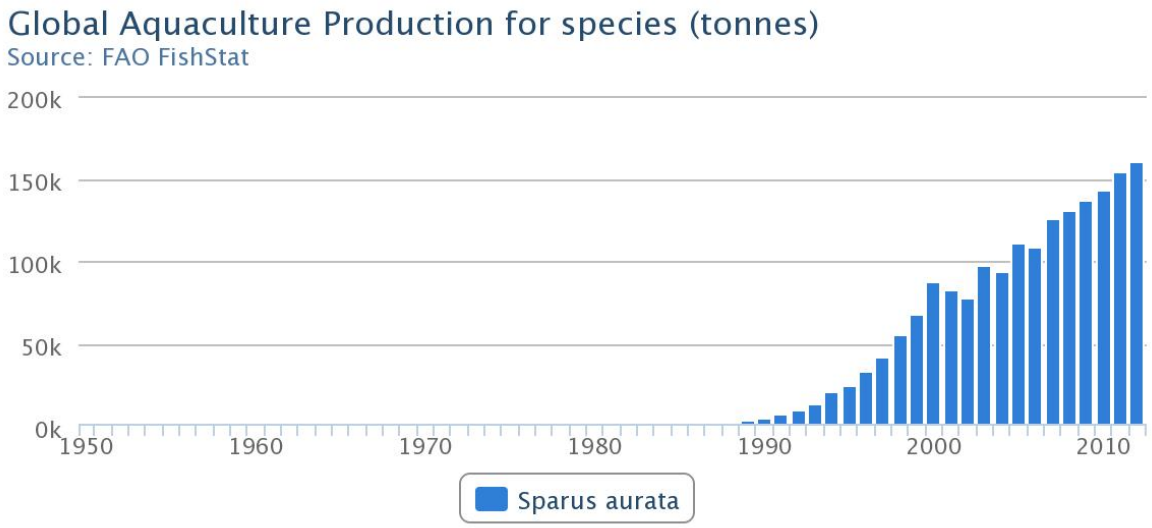

Fig. 2. Evolution of world aquaculture production of gilthead seabream in MT. From (FAO, 2014)

During the last years, there have been important advances in gilthead seabream productivity, caused by developments in production technologies, such as feeding systems automation, harvesting procedures, health management and diets improvements (Basurco *et al.*, 2011). Gilthead seabream can be produced in coastal ponds and lagoons, under extensive and semi-intensive systems but is mainly farmed intensively in sea cages at average densities of 15-25 kg m^{-3} with a food conversion ratio (FCR) of 1.5-2.0 (Basurco *et al.*, 2011). The farming period varies with location and water temperature, but typically it takes 18 to 24 months for a specimen to reach 400 g from hatched larvae, with the commercial size varying between 250 g and more than 1,5 kg (Basurco *et al.*, 2011). The gilthead seabream industry can be described as a sector that is entering its mature

phase but still needs a more efficient production to increase fish growth or to reduce production costs (Colloca & Cerasi, 2015).

1.3 Quality of the aquaculture product

The end quality of farmed fish must achieve a certain number of requisites in order to be commercialized. In general, the final product has a good quality stamp when it promotes the consumer's health and meets their demands and expectations. Fish quality is a broad concept to define, since it results from the combination of different attributes, such as the nutritional value and organoleptic characteristics of the flesh, but also welfare considerations (Poli *et al.*, 2005; Grigorakis, 2007). The three principals elements that influence the consumer willingness to eat a certain fish are the taste, the flavour and the colour (Grigorakis, 2007).

One of the handicaps of aquaculture is the consumer's preference for wild fish rather than the farmed ones (Grigorakis *et al.*, 2003). Nevertheless, in a survey, Greek consumers admitted to be unaware of the requirements of farmed fish welfare, packaging, distribution and quality standards, while about 50% of Greeks were unable to distinguish a farmed fish form a wild one, either pre of at consumption (Vlachos, 2013). The prejudice against aquaculture fish has to be stopped once the world's population is rising and farmed fish need to accomplish world's nutrition needs.

Muscle is the main product of aquaculture and consumers show a preference for fresh fish and with a firm texture (Valente *et al.*, 2013). Significant positive correlations have been found between muscle fibre size, density and texture characteristics such as chewiness and firmness (Hurling *et al.*, 1996; Johnston *et al.*, 2000; Periago *et al.*, 2005), muscle fatness (Grigorakis *et al.*, 2003) and collagen content. These parameters can be affected by a number of factors such as diet, photoperiod, temperature and exercise training (Salmerón *et al.*, 2013). Many other factors may affect flesh texture, depending on the fish species (biological condition of the fish), the method of catch or slaughter and/or culinary treatments (Rehbein & Oehlenschlager, 2009). After fish death, several biochemical changes linked to the onset and resolution of *rigor mortis* occur; before the onset of this step muscle is soft and elastic, in *rigor*, muscle becomes hard due to the contraction of the fibres and with its resolution the muscle becomes soft and less elastic (Rehbein & Oehlenschlager, 2009).

Appearance and texture of fish are affected by storage time, but they probably affect overall hedonic response less than do odour and flavour (Rehbein & Oehlenschlager, 2009). The most common texture failings are muscle softening and gaping forming caused by pre- and post-mortem treatment. These problems are normally

associated with the changes in chemical compositions and the degradation of muscle proteins (Cheng *et al.*, 2014).

1.4 Muscle structure and function

The general lateral muscular structure of the fish differs greatly from the structure of the terrestrial vertebrates. Fish has a segmented fillet with myotomes separated by a thin layer of connective tissue called myoseptum (Katz, 2002) (Fig. 3). Connective tissue is also present between the right and left side of the muscle section and horizontally separating the upper and lower muscle part of the body; this tissue represents only a small percentage of the total muscle weight, unlike in mammals (Bone, 1979).

Throughout the body, the myotomes are present in an oblique pattern perpendicular to the long axis of the fish, from the skin to the spine, with the inner edge closer to the front of the body (anterior) and the outer edge nearer the tail (posterior), which makes the characteristic W-shape look along the body fish (Van Leeuwen, 1999; Katz, 2002). Each myotome cells comprises several so-called muscle fibres that are oriented approximately parallel to the body axis, although this distribution varies in a deeper part of the muscle tissue where the muscle fibres are arranged in a helical way, forming angles of up to 40º to the longitudinal axis of the body (Alexander, 1969). This anatomy is ideal for the muscle movements necessary to propel fish through water during swimming activity (Palstra & Planas, 2012) (Fig. 3).

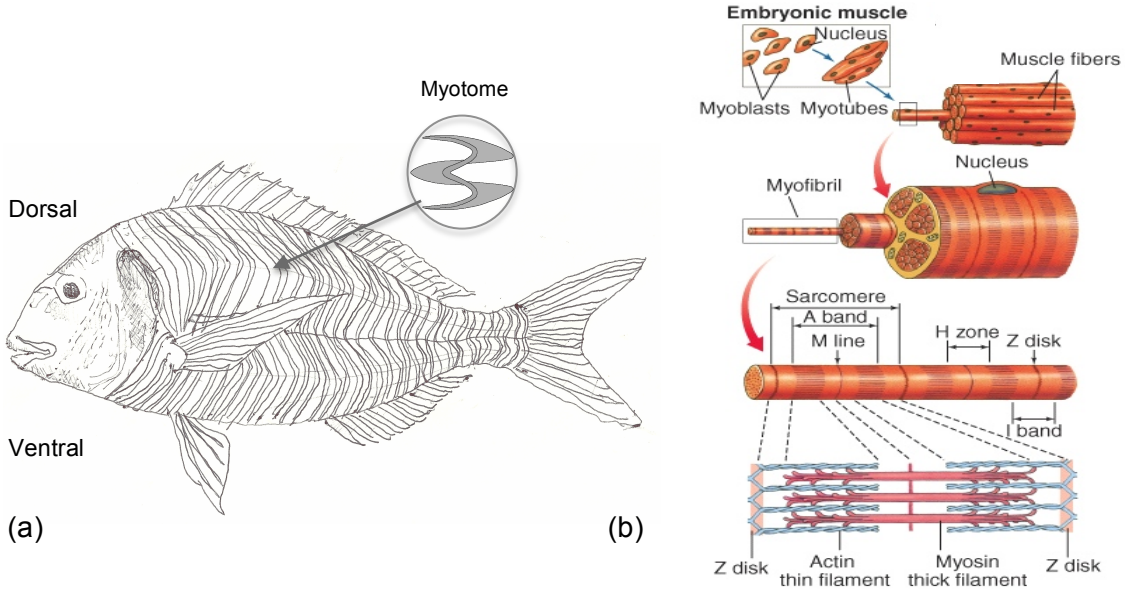

Fig. 3. Myotomal muscle in teleost fish. Myotomes as they appear in the lateral view of fish (a) (adapted from Bone, 1979) and a structure of vertebrate muscle fibre (b) adapted from (Randal *et al.*, 2002).

1.5 Muscle fibre types

The axial muscle of teleost has distinct fibre types, anatomically separated in two discrete layers: subdermal red muscle, also known as “slow twitch”, within slow oxidative metabolism, and deep white muscle, also known as “fast twitch” within fast glycolytic metabolism. In the transitional zone between slow-red and fast-white muscle, an intermediate pink muscle, can also be present. The relative amount of pink muscle fibres differs between fish species (Bone, 1979). Fast-twitch white muscle fibres comprise the major part of fish skeletal muscle (> 70%). These fibres have the largest diameters (50 – 100 μm or even higher) compared to slow-twitch red fibres (25 – 45 μm), depending on the locomotion mode (Sänger & Stoiber, 2001). The proportion of both muscle fibres is related to fish ecology, with active pelagic families having a higher proportion of slow fibres compared to demersal species, like the *Sparidae*, that use their fins as a primary mean of locomotion, and are almost entirely composed of fast muscle (Videler, 1993; Altringham & Ellerby, 1999).

These muscle fibres are multinucleated cells formed by the fusion of many cellules (myoblasts). Each fibre contains several parallel microscopic cylindrical myofibrils divided into identical contractile units, called sarcomeres and surrounded by a plasma membrane, the sarcolemma (Videler, 1993). The sarcomeres include two types of myofilaments, made of proteins called actin (thin) and myosin (thick), which cause the contractile activity of the muscle. These filaments are interconnected by disc-like structures known as Z-lines, giving a striated appearance upon microscopic examination (Videler, 1993).

In addition to myosin and actin filaments, many other proteins are present in the sarcomere structure (Fig. 4). Among them, for example myosin aggregates are connected to the Z-line through elastic titin filaments, from the Z-Disc up to the M-line. Besides the Z-Disc interconnections, sarcomeres are linked through intermediate filaments composed of desmin. There are also present in the fast muscle, cytoskeletal structures that connect the M- and Z-lines and the sarcolemma. These structures known as costameres, are composed of proteins like spectrin, vinculin, α-actinin, dystrophion, talin and filamin that bind to actin and integrin associated to membrane (Garcia de la serrana *et al.*, 2012). Integrins are connected to the extracellular proteins matrix such as collagen and fibronectin.

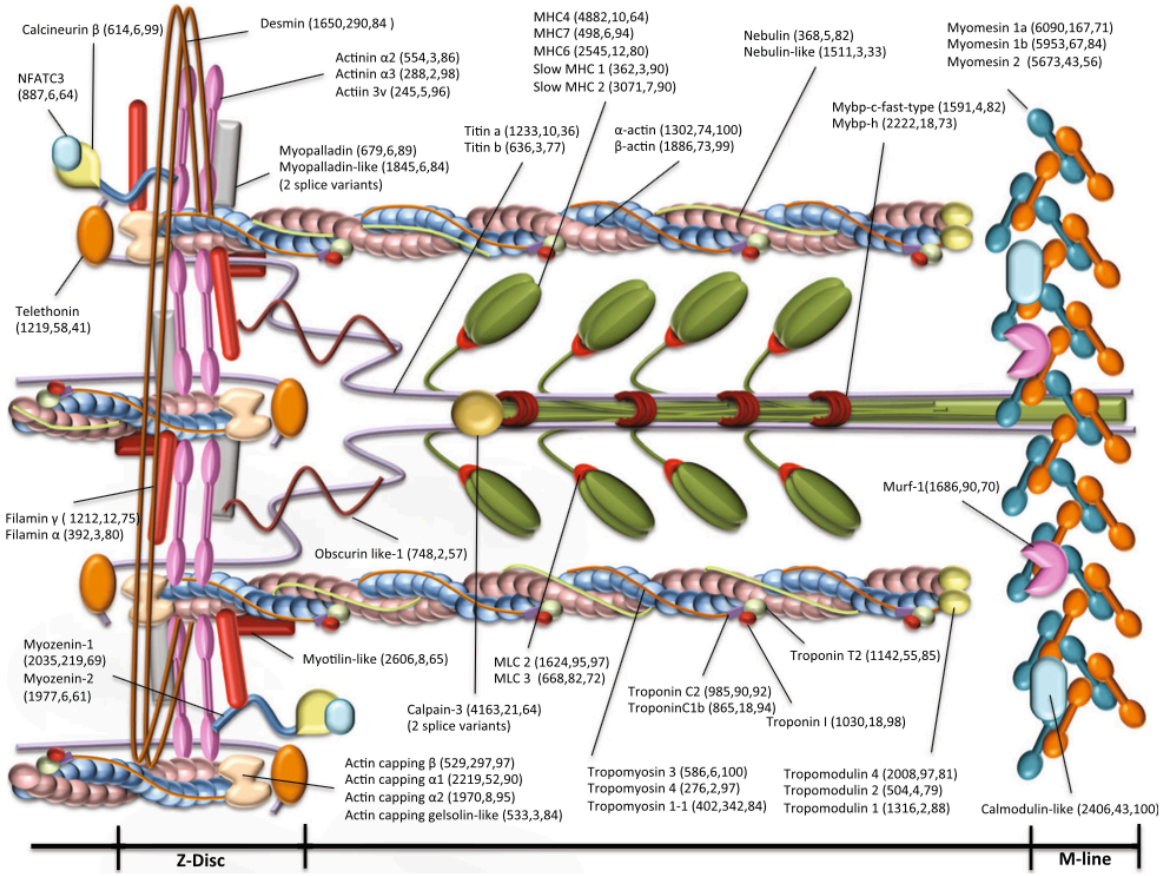

Fig. 4. Sarcomeric proteins genes represented in the fast muscle transcriptome of the seabream (*Sparus aurata)*. From (Garcia de la serrana *et al.* (2012).

Fish muscle contains approximately 18% protein, about 80% water in lean species and from 74-80% in lipid rich species, within lipids varying from a few percentage (i.e. Atlantic cod) to more than 20% (i.e. Atlantic salmon). The remain 2% constitute other nutrients (glycogen, minerals and vitamins) (Espe, 2008). Myofibrillar proteins are the most abundant ones (> 50%) with myosin and actin representing 27% and 11%, respectively of muscle protein composition. Myosin is a motor protein that interacts with actin filaments, using the energy from adenosine triphosphate (ATP) hydrolysis producing movement. This happens when a nerve impulse causes a release of calcium (Ca^{2+}) from the sarcoplasmic reticulum to the myofibrils. The Ca^{2+} concentration increases around the active enzyme site on the myosin filaments activating the enzyme ATP-ase. This ATP-ase splits the ATP present between the actin and myosin filaments, causing a release of energy. ATP hydrolysis is required for breaking the link between actin and myosin. Energy is necessary for each cycle of contraction, allowing the actin filaments to slide between the myosin filaments in telescopic way, thus contracting the muscle fibre (Fig. 5). The muscle returns to relaxed phase when the reaction is reversed (Huss, 1988). Four

proteins associated with the actin filament regulate contraction: tropomyosin and troponins C, I and T, Cytosolic Ca^{2+} levels influences the position of theses filaments on the actin filament, which in turn controls myosin-actin interaction (Purves *et al.*, 2003).

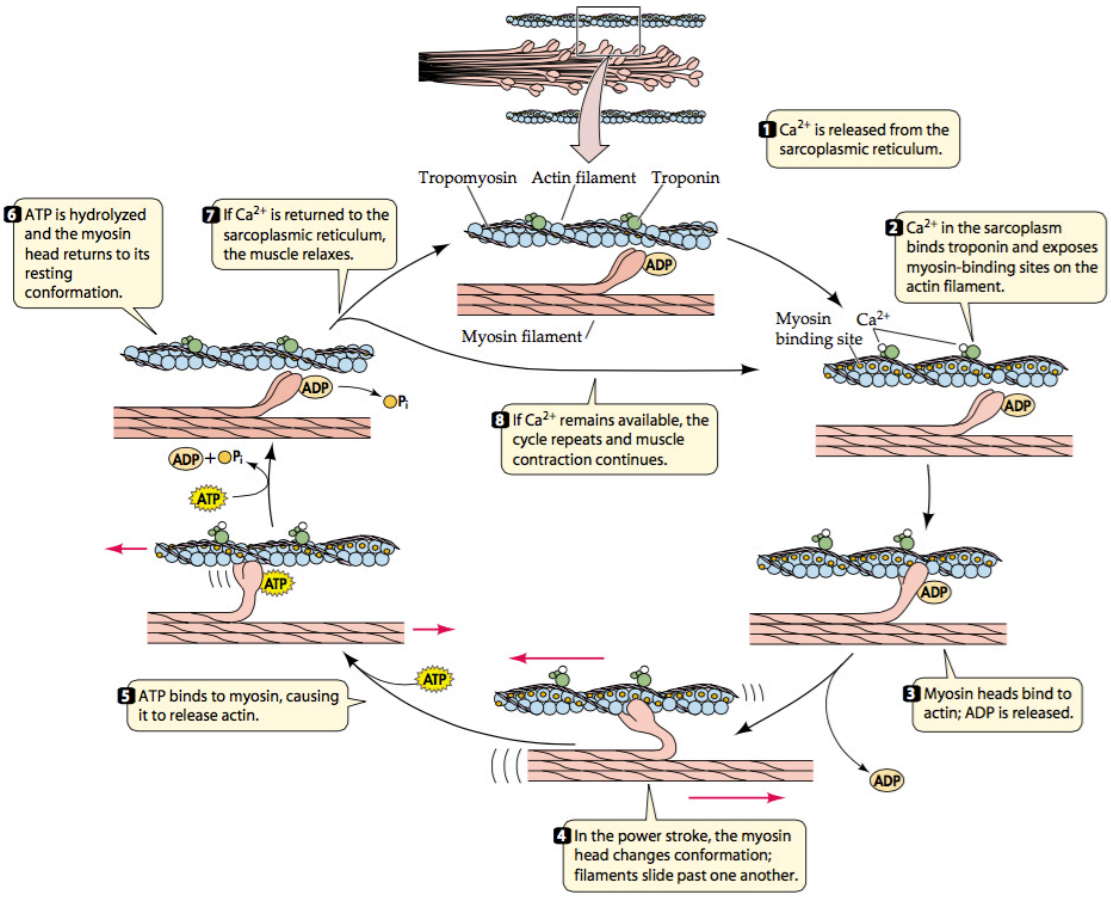

Fig. 5. Pathways contraction of muscle fibres. From (Purves *et al.*, 2003).

Normally, red muscle fibres have more capillaries, mitochondria and higher levels of lipid precipitations and myoglobin compared to other fibre types, which is responsible for its reddish colour (Bone, 1979). They also have relatively high concentrations of respiratory and citric acid cycle enzymes, required for energy generation during slow aerobic swimming, fuelled mostly by lipids and/or carbohydrates. The fast-twitch white muscle is generally used at high swimming speeds and is fuelled by phosphate hydrolysis followed by the activation of anaerobic glycolytic complexes. Resulting in a strong contraction but easily fatigued due to the accumulation of lactic acid in the white muscle, (recovery period) (Sänger & Stoiber, 2001).

Thick, non-contractile proteins, comprising mainly the slow isoform of myosin heavy chain, characterize red muscle fibres and it relies upon reduction in degradation rates rather than increase in synthesis rates. Fast-twitch skeletal muscle fibres have less

dense non-contractile proteins, faster myosin ATP-ase enzyme, and they rely more on increasing protein synthesis rates to increase cross-sectional fibre size (Ventura-Clapier *et al.*, 2002; Kraemer *et al.*, 2013).

It is known that the skeletal musculature is a high-energy demand tissue. In this sense creatine kinase (CK) is a key enzyme of the energy metabolism in cells and tissues with high-energy demands. CK catalyses the reversible reaction of the energy transfer pathway known as the creatine kinase/phosphocreatine (CK/PCr) energy shuttle, that provides immediate replenishment of ATP via high-energy phosphate compounds (Turner & Gant, 2014). CK, continues to have an important role after the slaughter of animals delaying the muscle pH decline and the onset of *rigor mortis* (Daroit & Brandelli, 2008).

The type of metabolism is another important feature of skeletal muscle fibres: a rapidly contracting muscle (glycolytic muscle) needs a large amount of energy within a short period of time. Large energy stores like phosphocreatine (PCr) can rephosphorylate ATP at rates high enough to cope with the muscle needs (Fig. 6). Once the energy reserves are exhausted the speed of contraction drops, and the energy is replenished by oxidative metabolism and glycolysis, Ventura-Clapier *et al.* (2002) designated this mode of contraction as “twitch now, pay later”, which is found mainly in locomotion muscles (Fig. 6A). The metabolic system used in obtaining ATP for the myosin motor of each muscle fibre is dependent upon the exercise stress (Fig. 6). In oxidative muscle, mitochondrial respiration is under the control of a specific mitochondrial CK (m-CK), where m-CK converts the newly produced ATP into PCr in the intermembrane space, while in glycolytic muscles such restriction is absent, revealing a tissue-specific regulation of mitochondrial function, both mitochondria and glycolytic complexes participate in the replenishment of the PCr pool (Fig. 6B)

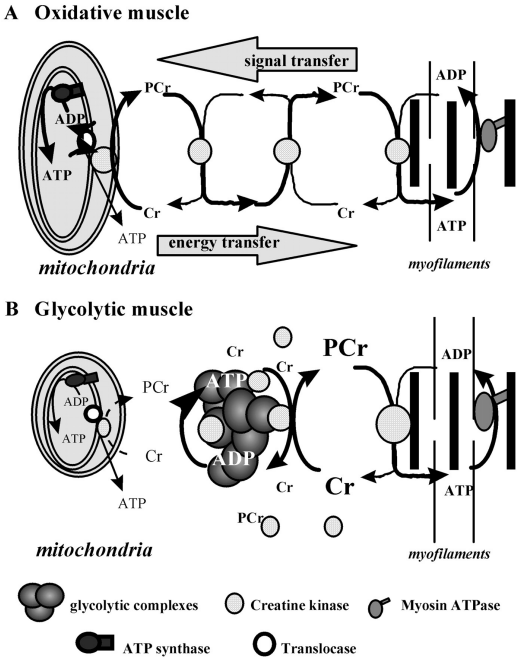

Fig. 6. The creatine kinase system in muscle cells. From (Ventura-Clapier *et al.*, 2002)

- *A*: in oxidative muscle, phosphocreatine (PCr) is synthesized in mitochondria owing to the localization of mitochondrial CK close to the translocase. PCr is transferred to bind cytosolic CK in myofilaments through near equilibrium reaction in the cytosol. In myofilaments, bound CK rephosphory-lates the ADP produced by myosin ATPase.
- *B*: in glycolytic muscles, the large pool of PCr serves as a spatiotemporal buffer for ATP and bound CK rephosphorylates the ADP produced by the ATPase. Mitochondria and glycolytic complexes participate in the replenishment of the PCr pool.

1.6 Muscle development and growth

Skeletal muscle is the final product of aquaculture as is the edible part of the fish (fillet). Understanding the mechanisms that regulate muscle development and growth is very important for the aquaculture industry in order to obtain and select fast growing fish and provide a final texture to fulfil consumer's expectations.

The skeletal muscle derives from the somites formed from the paraxial mesoderm in a rostral to caudal progression; this somatic growth reflects an increase in body size or weight gain controlled by both endogenous (hormonal-induced) and extrinsic (nutritional or environmental) signals (Company *et al.*, 2001; Pérez-Sánchez *et al.*, 2002). The axial musculature of the teleost occupies 60–70% of their body thus skeletal muscle is the largest organ system in fish. In gilthead seabream the axial musculature or fillet represents approximately 65% of body mass (Salmerón *et al.*, 2013). The major function of this contractile tissue is swimming activity (Espe, 2008).

The recruitment of new muscle fibres in fish continues throughout lifetime unlike in mammals, in which fibre recruitment of new muscle fibres ceases at the postnatal period (Johnston & Cole, 1998). In teleost fish, skeletal muscle formation involves the recruitment (hyperplasia) and enlargement of muscle fibres (hypertrophy and elongation) (Valente *et al.*, 2013). Through this process three different phases can be recognized in several teleost. The first phase occurs during embryonic period (embryonic myogenesis), mononucleated myogenic precursor adjacent to the notochord (adaxial cells) undergoes a mediolateral migration (Fig. 7a) generating a superficial slow fibre monolayer (primary myotome) underneath the dermomyotome (Fig. 7b) (Georgiou, 2013). The expression of slow and fast muscle fibre contractile proteins begins when the adaxial cells start differentiating (Rescan, 2008) and as soon as the cells are incorporated into the somite, they elongate and start differentiating. These cells develop into slow fibre type under the influence of sonic hedgehog (Shh) signalling by the notochord (Valente *et al.*, 2013), while myogenetic differentiation depends on the coordinated action of the myogenic regulatory factors (MRFs) which include myogenic factor 5 (*myf5*), myogenic differentiation 1 (*MyoD1*, also known as *MyoD*), Myogenin (*myog*) and myogenic regulator factor 4 (*mrf4*) combined with the action of the myocyte enhancer factor 2 (MEF2) family of MADS box factors (Bryson-Richardson & Currie, 2008; Ferri *et al.*, 2009).

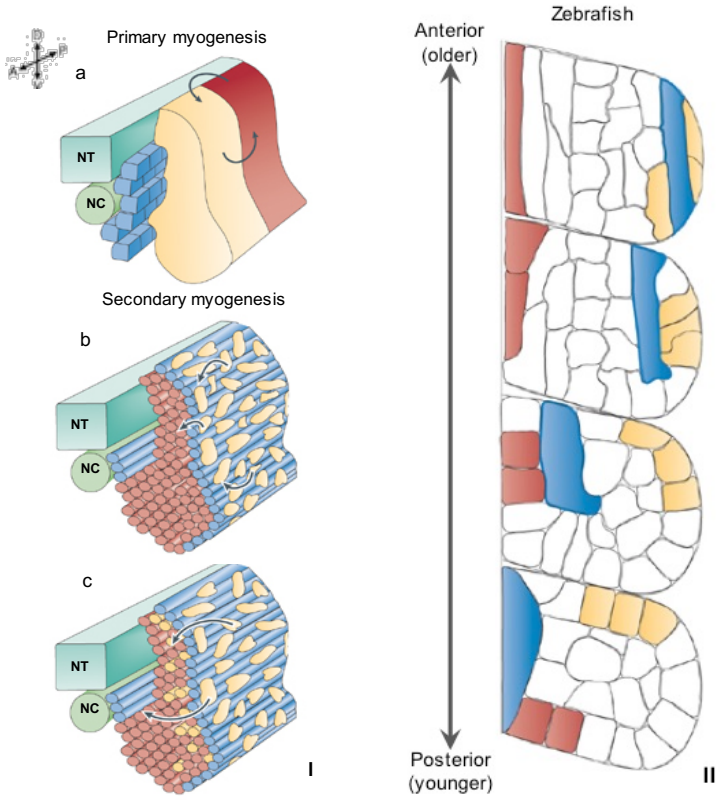

Fig. 7. Schematic of primary and secondary myogenesis (I) and somite-cell rotation in the zebrafish (II). (a) in the early fish embryo no dermomyotome is evident, instead an apparent myotome is separated anteriorly-posteriorly and undergoes rotation coincident with myogenesis; (b) the adaxial cells (blue) are the first fibres to differentiate in myotome with slow-muscle (blue) migration. The cells of the posterior compartment will differentiate to form the primary myotome; (c) The majority of the adaxial cells migrates to the lateral extent of the myotome forming the slow muscle (blue), and the fast muscle cells differentiate behind the migrating cells. Some adaxial cells (the muscle pioneers) remain at the notochord. Somite rotation form the anterior cells of the somite (yellow) moving lateral to the slow muscle to form the dermyotomal-like external cell layer, these cells contribute to secondary and adult myogenesis. NT, neural tube; NC, notochord; A, anterior; D, dorsal; P, posterior; V, ventral. Adapted from (Bryson-Richardson & Currie, 2008).

Production of new muscle fibres in discrete zone (stratified hyperplasia) is the second phase following embryonic hyperplasia and is especially intense in areas of small diameter fibres at the dorsal and ventral apexes (Georgiou, 2013). According to Valente *et al.* (2013), stratified hyperplasia has been identified in many species and is the major source of new fibres during early postembryonic and late embryonic growth.

The third and last phase of muscle differentiation and growth starts in the larvae and continues into adult stages. A hyperplastic process appears throughout the myotome surface involving newly formed muscle fibres, scattered between the existing ones, this process is known as mosaic hyperplasia due to the mosaic-like appearance of fibres of different ages and diameters (Rowlerson & Veggetti, 2001; Valente *et al.*, 2013). Mosaic hyperplasia is the main mechanism for fast fibre number expansion in juvenile and adult stages in most species, continuing until approximately 40-50% of the maximum fish length

(Johnston *et al.*, 2009). Mosaic hyperplasia can occur alongside with stratified hyperplasia, with mosaic hyperplasia having a precocious onset and contributes to a fast somatic growth, as seen in brown trout (Steinbacher *et al.*, 2007). At this phase the new cells fuse to form additional fibres or are absorbed by existing fibres as they expand in diameter (hypertrophic growth), and the maximum muscle fibre diameter can vary with body mass, activity patterns and metabolism (Johnston *et al.*, 2003; Johnston *et al.*, 2004). When the maximum fibre number is reached, myotube formation is inhibited unless the muscle is injured, suggesting the existence of a mechanisms that inhibits myotube formation in undamaged muscle in fish that are greater than approximately 40% of their maximum length (Rowlerson *et al.*, 1997). This last phase is very important for aquaculture species, since is the main contributing phase to the growth of skeletal muscle (Johnston, 2006; Valente *et al.*, 2013).

The formation of the skeletal muscle tissue, is common to all vertebrates and consists of a serial complex steps involving the specification, proliferation, differentiation, migration and fusion of precursor cells in order to form multinucleated muscle fibres (Valente *et al.*, 2013). Muscle formation (myogenesis) is mediated by various genes, namely the highly conserved basic/helix-loop-helix (bHLH), MRFs, that play an essential function in myogenic lineage determination and muscle differentiation (Rescan, 2001). MRFs activate muscle-specific transcription through binding to the Enhancer-box (E-box), which is a short sequence present in the promoter of numerous muscle genes (Rescan, 2001). *MyoD* and *myf5* are expressed in mesodermal cells committed to a myogenic fate, and play redundant roles in establishing myoblast identity, whereas *myog* and *mrf4* are involved later, initiating and maintaining the muscle differentiation program (Rescan, 2001; Buckingham & Vincent, 2009). Normally in fish, genes occur in pairs as the result of an ancient whole genome duplication after the Actinopterygian/Sarcopterygian (Jaillon *et al.*, 2004). In teleost like gilthead seabream, two paralogues of *MyoD* (*MyoD1* and *MyoD2*) have been described (Macqueen & Johnston, 2006; Andersen *et al.*, 2009).

Stem cells divide asymmetrically producing cells that become committed to a myogenic cell fate guided by the MRFs (*MyoD*, *myf5* and *mrf4*) (Valente *et al.*, 2013). After a first proliferation phase, the daughter cell generates the myogenic progenitor cells (MPCs) population that exits the cell cycle to initiate the terminal differentiation program. During the differentiation phase, myocytes fuse to form myotubes and subsequently mature and form muscle fibres under the influence of *myog*, *mrf4* and *Mstn*. Nuclear accretion (additional nuclei absorbed by the mature fibres) occurs as fibres increase in diameter and length in order to maintain the nuclear to cytoplasmic ratio within physiological levels (Fig. 8).

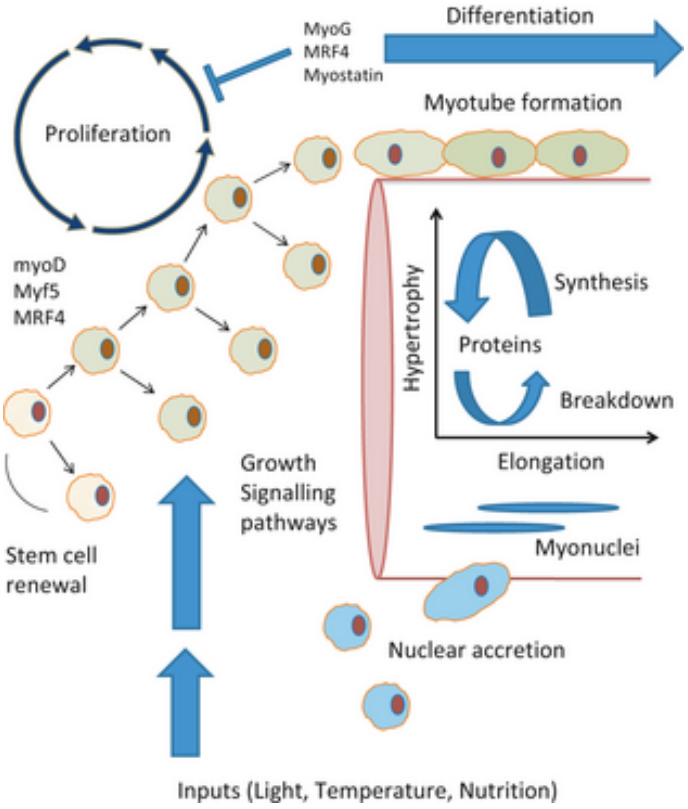

Fig. 8. A model of muscle growth in teleost fish. From (Valente *et al.*, 2013).

Myostatin (*Mstn*) is another gene that controls muscle growth, by controlling the proliferation of muscle precursor cells (Thomas *et al.*, 2000). Myostatin is a negative regulator of skeletal muscle growth during development and in the adult. Wang & McPherron (2012) found that myostatin inhibition in adult mice causes hypertrophy mainly by acting on myofibres rather than on muscle satellite cells (Wang & McPherron, 2012). The effect on muscle fibre number is likely to result from the activity of *Mstn* on myoblast proliferation and/or differentiation during development (Walsh & Celeste, 2005). *Mstn* effect on differentiation occurs through down-regulation of the MRFs (*MyoD*, *Myf5* and *Myog*) (Langley *et al.*, 2002). Two *Mstn* paralogues, *Mstn*1 and *Mstn*2, have been found in several fish species (Rescan, 2001).

1.7 Factors that influence muscle growth

1.7.1 Intrinsic factors

Muscle growth represents the balance between catabolic and anabolic components of protein metabolism (protein turnover, Fig. 8). Protein turnover plays an important role in the removal of defective proteins, and in the supply of amino acids as substrates for energy production and/or as precursors to synthesize new enzymes or structural proteins (Conceição *et al.*, 2008). The rates of protein synthesis and degradation in muscle fibres are carefully regulated. Body proteins are subject to continuous breakdown and replacement; it is known that skeletal muscle of many teleost undertakes an accelerated program of protein breakdown during seasonal periods of fasting and gonad maturation, when protein degradation overcomes protein synthesis, leading to atrophy. This system is complex and involves the ubiquitin-proteasome system and calpain proteases among others (Johnston *et al.*, 2011). It is known that even a small increase in protein synthesis or a small reduction in degradation if consistent over time, can result in an accretion of muscle in the organism (Valente *et al.*, 2013).

Some studies revealed that individual genetic characteristics might influence muscle growth patterns. For example, Johnston and McLay (1997) reported differences in myotomal fibre number between families in Atlantic salmon. Genetics also influence hormonal regulation, thereby potentially resulting in intra-specific variations in growth rate and a better "fitness" for aquaculture conditions. Also, Valente *et al.* (1998) determined the deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein levels in skeletal muscle of fast and slow growing strains of juvenile rainbow trout, observing that smaller cell size in the muscle of the fast growing strain is due to a higher DNA:protein ratio (Valente *et al.*, 1998). Latter morphometric study validated this biochemical result since smaller fast-twitch white fibre diameters and a greater number of small fibres were observed in the white muscle of this fast growing strain (Valente *et al.*, 1999).

Post-natal muscle growth in teleost fish involves the activation and proliferation of a set of quiescent myogenic precursor cells (MPCs), located at the periphery of the muscle fibers. In fish, muscle grows continuously via hyperplasic and hypertrophic mechanisms throughout life (juvenile and adult stages). Previous studies in Pirarucu (*Arapaima gigas*) revealed that the levels of MRFs and *Mstn* might be involved in a balance that controls hyperplasia and hypertrophy occurring during post-natal muscle growth, moreover *Mstn* did not appear to play a crucial role during early-juvenile stages (Carani *et al.*, 2014). Additionally the dietary inclusion of plant protein sources in rainbow trout lead to changes in expression of MRFs (*MyoD*), structural genes (*fast-MHC*) (Alami-Durante *et al.*, 2010a) and genes involved in muscle lysosomal proteolysis (cathepsin D)

(Alami-Durante *et al.*, 2010b), related with changes in skeletal white muscle cellularity (i.e fibre diameter).

Directly or indirectly, muscle growth is largely controlled by endocrine factors, like growth hormone (GH), insulin-like growth factor I (IGF-I), thyroid hormone and sex steroids (Mommsen & Moon, 2001). GH participates in all major physiological processes in fish, including osmotic balance, lipid, protein and carbohydrate metabolism and skeletal muscle tissue growth (Reinecke *et al.*, 2005; Wood *et al.*, 2005; De-Santis & Jerry, 2007). Previous studies in rainbow trout (Weatherley & Gill, 1982; Fauconneau *et al.*, 1997), coho salmon (Hill *et al.*, 2000) and Arctic charr (Pitkänen *et al.*, 2000) revealed that the supplementation of growth hormone (GH) promotes hyperplastic growth of these fish species.

Myogenesis is regulated by the myogenic regulatory factors (*MyoD*, *Myf5*, myogenin and *Mrf4*) and by endocrine signals from the growth hormone/insulin-like growth factors axis (Jiménez-Amilburu *et al.*, 2013). Jiménez-Amilburu *et al.* (2013) use in vitro cultured *S. aurata* myocytes to understand the role of endocrine factors (GH, IGF-I and IGFII) in MRFs, this study reported that IGF-II increased expression of genes involved in early muscle cell proliferation, namely *MyoD2* and *Myf5*. Also, IGF-I caused an increase on *Mrf4* and myogenin expression, both involved in the later stages of development corresponding to differentiation.

Calpains are cysteine proteases activated by Ca^{2+}, and play an important role in a wide range of cellular processes such as apoptosis, migration, cell cycle regulation, protein replacement, myogenesis and metabolisms among others (Goll *et al.*, 2003; Zhivotovsky & Orrenius, 2011; Campbell & Davies, 2012). There are fifteen different isoforms of calpains, which can be divided into: large or catalytic subunits and regulatory subunits or small calpastatin, which is specific inhibitor of calpain. Moreover, calpains can be grouped based on the presence or absence of calcium binding domain, “EF-hand”.

So there are calpains with “EF-hand”, known as classical calpains (calpains 1, 2, 3, 8, 9 and 11 to 14), which contains a C2-like (CL2) and a penta EF-hand (EF) domain plus the calpain-like protease (CysPc) domain and the remaining non-classical calpains (calpains 5, 6, 7, 10, 15 and 16) that lack both, the CL2 and PEF domains (Salmerón *et al.*, 2013). Finally, calpains can also be classified according to their location: ubiquitous calpains (1, 2, 13 and 14) and tissue-specific calpains (3, 8, 9, 11 and 12), which are expressed only in certain tissues, such as skeletal muscle (calpain 3), gastrointestinal tract (calpain 8 and 9) among others (Ono & Sorimachi, 2012).

Numerous proteins including cytoskeletal proteins, kinases, phosphatases, membrane associated proteins like receptors or ion channels, and some transcription factors have been described to be cleaved by calpains in *in vitro* essays (Goll *et al.*,

2003), and seem regulated by a specific ubiquitous inhibitor called calpastatin (Fig. 9) (Johnston *et al.*, 2011).

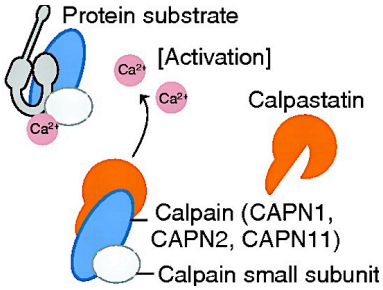

Fig. 9. The calpain family comprises a major cytosolic protein breakdown pathway. These calpains (1,2 and 11) regulate many other physiological processes like myoblast fusion. Ubiquitination comprehends the targeting of structural and regulatory muscle proteins for activation by proteases. Adapted from (Johnston *et al.*, 2011)

Calpains function as a heterodimer formed by the union of two subunits: a large subunit or catalytic (80 kDa) generally formed by calpain 1 or 2, also known as μ- or m-calpain respectively according with the Ca^{2+} concentrations required for activation (3-50μM and 400-800μM respectively) and a common small subunit or regulatory (28 kDa), the calpain s1 (Goll *et al.*, 2003). It is believed that the small subunit normally acts like a chaperone, which may help the large subunit to proper fold to make it active. It was demonstrated in mice that the lack of m-calpain or small subunit is lethal in embryos, while mouse without μ-calpain or calpastatin are healthy (Zimmerman *et al.*, 2000; Takano *et al.*, 2005; Dutt *et al.*, 2006).

The μ-calpain and calpastatin are important in mammalian production, composition and meat quality, because it has been described allelic variants (SNPs) associated with those characteristics. A single nucleotide polymorphism (SNP) is a molecular marker bi-allelic and co-dominant. Furthermore, even thought calpain 3 has been described as muscle specific, it was found a correlation between its expression levels and meat tenderness in some animal groups (e.g. cows and sheep) but not in others (e.g. pork) (Parr *et al.*, 1999). However, as calpain 3 can also degrade calpastatin and ubiquitous calpain, it can have a role in the regulation of the expression and proteolytic activity of other muscle calpains. Using C_2C_{12} cells as a model, Stuelsatz *et al.,* (2010) showed that *CAPN3* is involved in the regulation of the myogenic regulatory factors, *MyoD,* by inducing its destabilization and leading myoblasts to quiescence. Further studies are still needed to understand its physiological functions (Ono *et al.*, 2004).

In fish, members of the calpain family and calpastatins generated by differential "slicing", have been reported in different species, namely rainbow trout. Using different rainbow trout strains with distinct growth rates and fillet firmness and fed with two different

energy diets, Salem *et al*. (2005a and b) found that their expression can be modulated by nutritional status. Moreover, a lower expression of calpastatin was observed in the strained with the softest fillet texture (Salem *et al.*, 2005).

1.7.2 Extrinsic factors

Extrinsic factors, such as environmental inputs affect skeletal muscle growth, resulting in phenotypic changes linked with locomotion, metabolism and growth. This plasticity of the skeletal muscle, often involves structural changes in cellular organelles or supporting structures like capillaries (Sänger & Stoiber, 2001; Johnston, 2006). Gilthead seabream is susceptible to this, since their habitats often show complex temporal-spatial variations in temperature, salinity, oxygen content, pH, light availability and water flow.

Although phenotypic changes in muscle during embryonic or larval stages are usually irreversible due to their rapid ontogenetic development. Once fish reaches the adult stage this changes can be reversible. Factors related to other energy-demanding processes will have a role on energy potentially allocated to growth; for example growth is slowed during breeding or even stop when fish stop feeding (Johnston, 2006).

1.7.3 Diet

Diet is major factor affecting fish somatic growth. The goal of every aquaculture farmer is to obtain a fast growth, and this largely depends on sufficient dietary inputs of all essential nutrients. A good diet composition and an optimum-feeding regime are crucial for the quality of the fillet (Valente *et al.*, 2013).

Houlihan *et al.* (1993, 1995) revealed that an increase in net protein synthesis in muscle is linked to a larger dietary intake; therefore muscle growth is dependent on a positive balance between protein synthesis and degradation. Reduced ration size and fish held under conditions of severe nutritional restriction leads to atrophy of white muscle fibres and reduces muscle growth (Johnston & Moon, 1981; Rowlerson & Veggetti, 2001).

Protein fraction is considered the principal component affecting fish growth; usually fish require more protein than other vertebrates. This need seems to be associated with fish preference to use amino acids as an energy source rather than carbohydrates or lipids (Bowen, 1987). In gilthead seabream an optimum dietary protein level was estimated to be around 55% for fry and 45% in larger fish, suggesting a reduction of protein requirement along the lifecycle (Santinha & Gomes, 1996; Vergara *et al.*, 1996a; Vergara *et al.*, 1996b).

Lipids and carbohydrates do not have a role on fish growth as important as proteins do; however, they must be present in proper proportions to cover all the nutritional needs of each species to achieve an optimal growth. In gilthead seabream an optimum dietary lipid level of 15-16% was established, although later studies revealed that an increase to 22% in dietary lipid leads to higher weight gain; nonetheless no higher levels are advised as it may lead to liver abnormalities (Vergara & Jauncey, 1993; Vergara *et al.*, 1999). Regarding carbohydrates, Venou *et al.* (2003) observed that gilthead seabream performed better with diets including up to 40% of wheat starch compared with corn. This can be related with gilthead seabream natural diet (molluscs, particularly mussels), that have high carbohydrate levels, and they can be accessorily herbivorous as well (Basurco *et al.*, 2011).

1.8 Creatine metabolism

Creatine (Cr) is a nonessential amino acid derivative that is naturally found in highest abundance in vertebrate skeletal muscle (~95%) with most of the remaining stores found in the heart, brain and testes (Wyss & Kaddurah-Daouk, 2000; McFarlane *et al.*, 2001; Snow & Murphy, 2001). A typical total creatine pool in 70 kg human amounting to approximately 120g (Walker, 1979; McFarlane *et al.*, 2001; East, 2002). Creatine was firstly discovered in 1832 by a French scientist, Michel Eugène Chevreul, while he was investigating meat extract, and later a German scientist, Justus von Liebig (1847) chemically identified Cr as methyl-guanidino-acetic acid (relatively simple guanidine compound), confirming Chevreul's discovery (East, 2002; Wallimann, 2007; Kraemer *et al.*, 2013).

Cr is an important physiological compound as part of the adenosine triphosphate (ATP)/phosphocreatine (PCr) phosphate energy system. Cr and inorganic phosphate combine to form phosphocreatine and a greater Cr pool allows for higher concentrations and/or rates of PCr biosynthesis in the muscle (Kraemer *et al.*, 2013). The breakdown of PCr allows an increase and rapid biosynthesis of ATP, being PCr an immediate fuel reserve for the replenishment of ATP in the ATP/PCr energy system, therefore creatine is an important substrate supporting this system (Kraemer *et al.*, 2013) (Fig. 10).

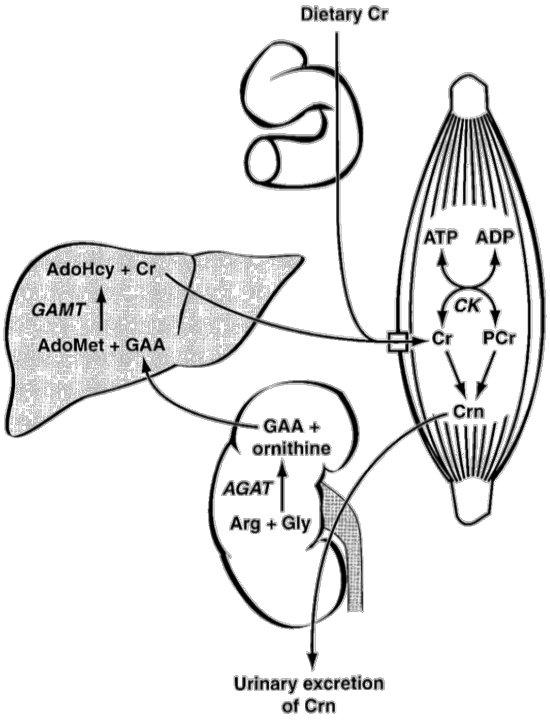

Fig. 10. Creatine metabolism. From (Wyss & Kaddurah-Daouk, 2000)

1.8.1 Creatine synthesis

The endogenous creatine synthesis occurs primarily in the liver from two amino acids by a two-step reaction of about 1g per day in humans but also at a small rate in the kidney and pancreas, from the amino acids glycine, arginine and methionine . The transfer of methyl group from S-adenosyl-methionine (SAM) is the irreversible reaction (committed step) during synthesis (Wyss & Kaddurah-Daouk, 2000) (Fig. 11). While arginine participates in the urea cycle, glycine is a precursor of purine nucleotides, and methionine contributes its methyl group to DNA and RNA (East, 2002). This process is regulated by dietary sources of creatine, implying that a high dietary Cr intake will result in a lower rate of endogenous synthesis. The first step of Cr synthesis begins with the transfer of the amidino group of arginine to glycine through the enzyme L-Arginine: glycine amidinotransferase (AGAT). The products of this activity include L-ornithine and guanidinoacetic acid, which is then methylated by SAM throught the enzyme S-adenosyl-L-methionine: N-guanidinoacetate methyltransferase (GAMT), resulting in the endogenous production of Cr (Kraemer *et al.*, 2013). Borchel and his team (2014) suggest that in rainbow trout, creatine synthesis takes place mainly in the skeletal muscle.

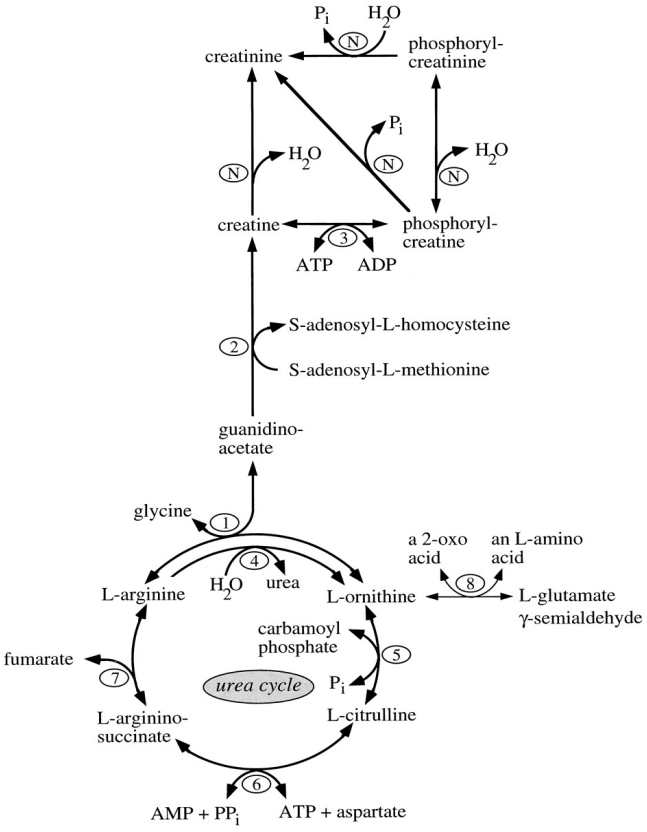

Fig. 11. Schematic representation of the vertebrate creatine metabolism: biosynthesis, degradation and phosphorylation/dephosphorylation. The numbers represent the respective enzymes: 1) L-arginine:glycine amidinotransferase (AGAT); 2) S-adenosyl-L-methionine:N-guanidinoacetate methyltrans- ferase (GAMT); 3) creatine kinase (East) 4) arginase (L-arginine amidinohydro- lase) 5) ornithine carbamoyltransferase; 6) argininosuccinate synthase; 7) argininosuccinate lyase (8) L-ornithine:2-oxo-acid aminotransferase (OAT); N) nonenzymatic reaction. From (Wyss & Kaddurah-Daouk, 2000).

Besides the endogenous production, muscular creatine stores are obtained from exogenous dietary intake both in humans and in fish (McFarlane *et al.*, 2001; Volek *et al.*, 2008). Creatine obtained from food has a high bioavailability in humans, allowing it to pass through the digestive tract and directly to the bloodstream (Volek *et al.*, 2008). Once in the bloodstream, Cr is taken up by the tissue (mostly skeletal muscle) or is excreted by the kidney, muscle uptake of Cr occurs via sodium-dependent Cr transporters with the muscle membrane (Kraemer *et al.*, 2013) (Fig. 12).

The storage of creatine can vary with different muscle types, more creatine is found in class II fast-twitch white muscle fibres (typically responsible for anaerobic energy) while less is found in class I slow-twitch red muscle (responsible for aerobic energy) (East, 2002). The white muscle has 31 % more PCr than red muscle (East, 2002).

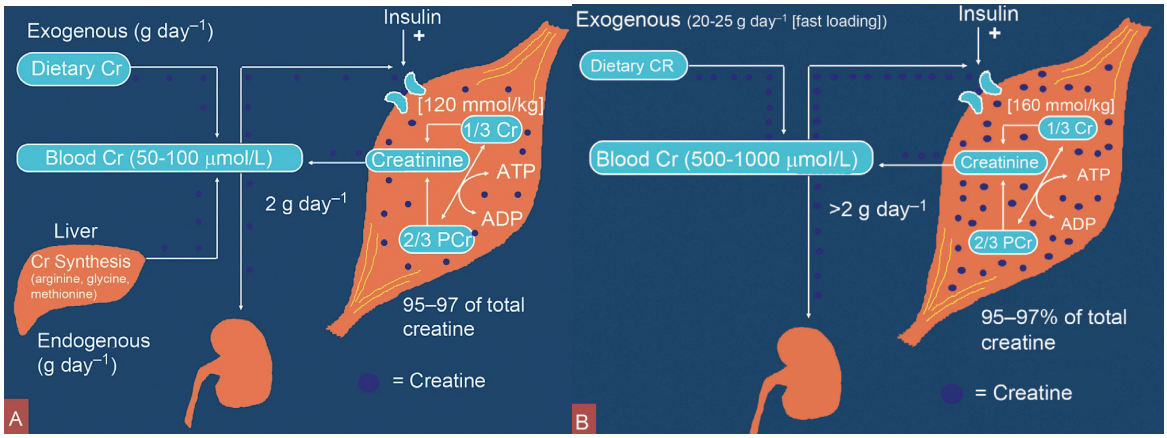

Fig. 12. Cybernetic interactions between creatine and muscle in humans. (A) Normal creatine dynamics without supplementation. (B) Creatine synthesis with supplementation. From (Kraemer *et al.*, 2013)

1.8.2 Creatine supplementation on skeletal muscle

Creatine kinases (CKs) catalyse the regeneration of ATP by transphosphorylation of energy-rich phosphate from phosphorylcreatine to the ADP produced in energy consuming processes like pumping of ions, muscle contraction, *etc.* (Wyss & Kaddurah-Daouk, 2000). In recent decades, however, a series of new discoveries have been aasocieated to creatine. For example, Cr analogues have proven to be potent anticancer agents acting synergistically with chemotherapeutics available in the market (Wyss & Kaddurah-Daouk, 2000). Oral creatine ingestion has also been shown to increase athletic performance, and in the 1990s athletes started to use it as a performance-boosting supplement (Wyss & Kaddurah-Daouk, 2000; Kraemer *et al.*, 2013).

It is well known that oral ingestion in Humans of Cr in form of meat, fish or via supplements will add to the whole body a Cr pool (Greenhaff *et al.*, 1994). Studies have shown that Cr ingestion in humans can significantly increase the amount of work that can be performed during repeated sessions of maximal exercise (Greenhaff *et al.*, 1994). During the initial loading phase of Cr supplementation, gains in body mass are typically attributed to an increase in water retention, caused by the osmotic activity of Cr; the increase in Cr concentration within the sarcoplasm of the muscle fibre draws water into the cell (Kraemer *et al.*, 2013).

Most of the studies focused on the ergogenic effects of creatine supplementation in conjunction with resistance exercise. Creatine supplementation has resulted increased type I, IIA and IIAB muscle fibre cross-sectional area compared with placebo after 12 weeks of heavy resistance (Volek *et al.*, 1997). It also leads to increased levels of type I and II myosin heavy chain (MHC) mRNA transcripts and protein content after 12 weeks of resistance exercise, suggesting that an increase in MHC synthesis may result in

increased muscle size after creatine supplementation (Willoughby & Rosene, 2001). It has been hypothesized that increased MHC gene expression induced by creatine supplementation is mediated by MRFs. In fact, *Mrf4* level was increased after creatine intake in combination with resistance training. Increased *Mrf4* and myogenin protein were correlated to muscle creatine kinase mRNA expression (Willoughby & Rosene, 2003).

Recent evidences show that creatine can effectively influence gene transcription. Safdar *et al.* showed that short-term creatine supplementation for 10 days in young men is able to increase the expression of numerous genes involved in osmotic regulation, glycogen synthesis and degradation, cytoskeletal remodelling, proliferation and differentiation of satellite cells, repairs and replication DNA, RNA transcriptional control and cell death (Safdar *et al.*, 2008).

Although most studies related with Cr have been carried out in humans, the effect on meat quality has also been studied. CK is a crucial enzyme for the *in vivo* energy metabolism of skeletal muscles from meat animals, and continues to have significance after the slaughter as well, because it delays the muscle pH decline and the onset of *rigor mortis.* Cr supplementation in pork diets prior to slaughter may affect post-mortem muscle metabolism and improve pork quality (James *et al.*, 2002).

The findings of Robert E. Young *et al.* (2007) on the effect of creatine supplementation in rat muscle performance support the argument that the beneficial effects of creatine on muscle mass and strength are due to an enhanced ability to train rather than a direct effect on muscle. The potential anabolic effects of creatine might depend on the adjustment of workout intensity during training.

The importance of the creatine system in fish has not been addressed so far. However it was demonstrated that fish muscle have a higher creatine content than mammalians (Hunter, 1929). Additionally McFarlane *et al.* (2001) found that exogenous creatine supplementation (dietary or injected) does not alter muscle creatine levels; nevertheless coincident with the Cr intake they observed an increase in endurance during a fixed velocity sprint test in rainbow trout. This study suggests that fish muscle creatine and PCr levels are less susceptible to manipulation than human muscle stores. This lack of creatine loading may be related to diet composition. The greatest source of creatine for carnivorous fish is the muscle tissue, as fish have a low capacity to endogenously synthetize creatine (Danulat & Hochachka, 1989). Therefor the lack of effect of supplemented creatine on muscle creatine content suggest that fish may already be at the upper range of their creatine storage capacity (McFarlane *et al.*, 2001).

2 Objectives

To our knowledge, no information on the effects of oral Cr supplementation on fish muscle cellularity is currently available. The aim of this Thesis is to contribute to a better understanding of the effects of Cr supplementation on *S. aurata* juvenile's muscular growth both through histological parameters (cellularity of the fast twitch muscle tissue) and molecular biology techniques (expression of muscle-related genes). The genes selected for this study are involved in myogenesis (*MRFs*, *MyoD* and *myog*), muscle structure and function (myosin heavy chain, myostatin) and muscle proteolysis (calpains). This study expects to correlate skeletal muscle cellularity and expression of muscle growth-related genes in relation with dietary creatine supplementation.

3 Material and Methods

3.1 Experimental diets

A control diet (CTRL), similar to commercial seabream feed, was formulated (49% protein and 23 $kJ.g^{-1}$) to fulfil the known nutritional requirements of the gilthead seabream. Three other different diets were prepared, by adding 2% Cr, 5% Cr and 8% of Cr monohydrate to control diet similar to the control diet (Table 1). All diets were formulated to fulfill the known nutritional requirements of the species and manufactured by SPAROS (Faro, Portugal).

Table 1. Ingredients and proximate composition of the control diet

Ingredients, %	CTRL
Fishmeal LT[a]	10.00
Fishmeal 60[b]	10.00
Porcine blood meal	5.00
Soy protein concentrate[c]	10.00
Wheat gluten[d]	10.00
Corn gluten[e]	7.25
Rise protein concentrate	3.50
Soybean meal[f]	10.00
Rapeseed meal	4.00
Wheat meal	12.00
Fish oil[f]	14.50
Vit & Min Premix[g]	0.15
Soy lecithin[h]	2.00
Antioxidant	0.40
Dicalcium Phosphate[i]	0.50
L-Lysine[j]	0.50
DL-Methionine	0.20
Creatine (g/kg)[K]	0.00
Proximate composition	
Dry Matter (DM) (%)	95.39±0.04
Crude protein (%DM)	49.28±0.14
Lipid (%DM)	20.37±0.31
Ash (%DM)	8.39±0.06
Gross energy (kJ/g DM)	23.43±0.07

[a] Peruvian fishmeal LT: 71% crude protein, 11% crude fat, EXALMAR, Peru.
[b] Fish by-products meal: 540 g Kg^{-1} CP, 80 g kg^{-1} CF, COFACO, Portugal.
[c] Soycomil P: 65% CP, 0.7% CF, ADM, The Netherlands.
[d] VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.
[e] GLUTALYS: 61% CP, 8% CF, ROQUETTE, France.
[f] Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL, Portugal.
[f] Henry Lamotte Oils GmbH, Germany
[g] PVO40.01 SPAROS standard premix for marine fish, PREMIX Lda, Portugal.
[h] Yelkinol AC (65% phospholipids): 750 g Kg^{-1} CF,ADM, The Netherlands
[I] Dicalcium phosphate: 18% phosphorus, 23% calcium, Fosfitalia, Italy.
[j] L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France
[K] Creatine monohydrate: Sigma-Aldrich, USA

Main ingredients were grinded (below 250µm) in a micropulverizer hammer mill Hosakawa, model #1 (Hosokawa Micron Ltd., United Kingdom). These triturated ingredients were then mixed accordingly to the target formulation in a Double-helix Mixture TGC, model 500L (TGC Extrusion, France), to attain a basal mixture (no oils were added at this stage). All diets were manufactured by extrusion (pellet size 5.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105 – 110ºC. Upon extrusion, all batches of extruded feeds were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 hours at 60ºC. After this process, pellets were left to cool at room temperature, and subsequently the creatine was mixed with fish oil fraction in concentrations (2,5 and 8%) according to each target formulation and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands) respective mixture. Throughout the duration of the trial, experimental feeds were stored at room temperature.

3.2 Animal growth conditions

The current trial was conducted according to the European Economic Community animal experimentation guidelines, Directive of 24 November 1986 (86/609/EEC) at Ramallhete, CCMAR facilities (Centre of Marine Sciences of Algarve), from July to September 2014. Triplicate groups of 24 gilthead seabream (initial body weight: 173 ± 2.4 g) were randomly distributed by 500 L tanks and hand-fed *ad libitum* twice a day (except Sundays) each experimental diet per 69 days. Sea water was supplied at 2l/min (mean temperature 23.3ºC ± 0.90; mean salinity 37 ± 0.39) in a flow through system with artificial aeration (mean dissolved oxygen above 5 $mg.L^{-1}$). All physical and chemical water parameters were evaluated during the experiment to ensure the experimental design.

3.3 Sampling

At the end of the experimental trial, all fish were deeply anaesthetized in an aqueous solution of MS-222 (Sigma, Switzerland), and individually weighted. These data was used to calculated daily growth index [DGI = $100x(FBW^{1/3} – IBW^{1/3})$/trial duration (days)]. Six fish from dietary treatment were individual weighted (g) and measured for total standard length (cm) and were killed by decapitation under a cork board on ice. Their fins were then cut and fish were softly scaled on both sides. A cross section fillet with skin (2-3 mm thick) was taken immediately before the dorsal fin position (Figure 13a). The dorsal

area of each fillet was then quickly photographed (with scale reference) and properly labeled, for later determination of the cross section area. Then the skin and the red muscle of each fillet were then removed and four representative samples were collected from the right part of the fillet (Figure 13b). Each small piece of muscle (0,5x0,5 cm) was immediately placed in a cryoprotective embedding medium – OCT (Thermo Scientific™ Shandon™ Cryomatrix™) and snap frozen in isopentane cooled by liquid nitrogen and stored at – 80ºC for later morphometric study.

A second cross sectional fillet (Figure 13a); fillet B was taken and 2-3 g of white muscle samples (right fillets, without skin and red muscle) were taken and stored in a RNA*later*™ solution (Sigma-Aldrich, USA) overnight at 4ºC. The excess solution was then discard and the samples stored at – 80ºC for posterior molecular biology analysis.

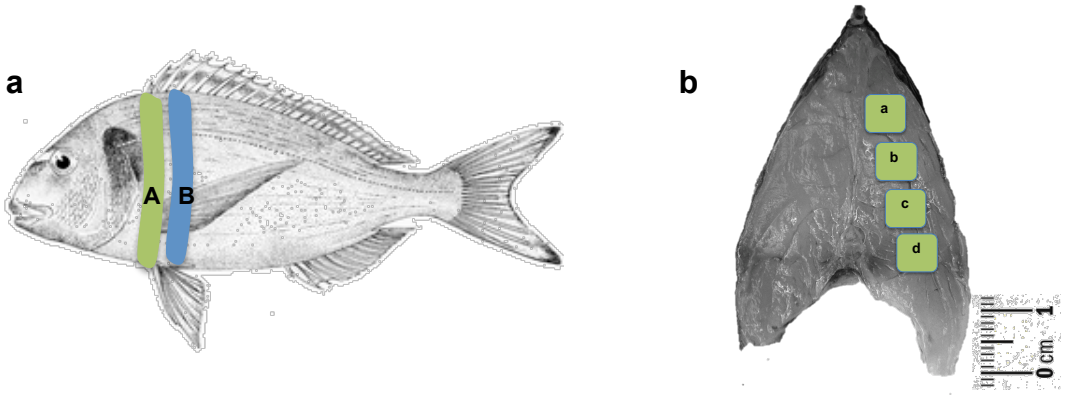

Fig. 13. a) Fillet sampling area for histology parameters (A) and for molecular biology analysis (B) (Adapted from Colloca & Cerasi, 2015) and b) selected four sampling dorsal areas (a-d) for muscle cellularity evaluation.

3.4 Morphometric procedure

The morphometric study was made using an interactive image analysis system (Olympus Cell*Family), working with a live-image captured by CCD-video camera (ColorView Soft Imaging System, Olympus) and a light microscope (BX51, Olympus, Japan. Muscle total dorsal muscular area (DMA) (mm^2), was computed by the software after demarcating half of the physical limits of the whole dorsal section, without considering any red muscle area. These measurements were based on the photo taken at sampling time, and an estimate of the total dorsal area was obtained by doubling the computed value.

Transversal white muscle sections were cut at 7 µm in a cryostat CM 1950 (Leica Microsystem GmbH, Wetzlar, Germany) from each block (a-d) and mounted on polysine adhesion slides. Sections were stained with haematoxylin-eosin (Merk, Whitehouse Station, NJ, USA) before placing a cover slip, and left to dry. The relative number (density) of white muscle fibres per unit area N_A(nº/mm^2), was estimated as follows: N/area = Σ

N(fibres) / Σ [a (sampled field)] where ΣN (fibres) is the total number of fibres counted over the sampled fields in the sections (a-d), and "a" is the total area of the fibre counting fields. The total number of white muscle fibres per dorsal cross section (N) was estimated as follows: N (fibres) = N_A (muscle fibres) x DMA (muscle) where N_A is the number of white muscle fibres per unit area (mm^2) and DMA the dorsal muscle area. From each fish, the physical limits of a minimum of 700 white muscle fibres (from the four blocs a-d) were circumscribed using a 20x objective to determine mean fibre area [$\bar{a}$ (μm^2)]. The corresponding mean diameter was calculated assuming that all fibres were circular.

3.5 RNA extraction and cDNA synthesis

White muscle samples were disrupted with a PureZol solution (Bio-Rad Laboratories), using Precellys® 24 lysis/homogenizer (Bertin Technologies, France). Total RNA was extracted using the Ilustra RNAspin Mini RNA isolation kit (GE Healthcare UK Limited), including an on-column DNAse digesting step, according to the manufacture's instructions. RNA quantification and quality were evaluated by absorbance at 260 and 280 nm using the Take3 Micro-Volume plate (Take3, Biotek, Germany) and the Gen 5 software (BioTek, USA), and the values were within the expected ratio of 1.8 – 2.2, indicating high RNA purity. RNA integrity was verified by the banding pattern of 28S:18S ribosomal RNA in 1% TAE (w/v) agarose gel electrophoresis stained with GelRed (Biotium, Hayward CA, USA).

For complementary deoxiribonuclein acid (cDNA) synthesis, 750 ng of total RNA were transcribed for all samples, with the iScript™ Reverse Transcription Supermix for real-time polymerase chain reaction (RT-qPCR) (Bio-Rad Laboratories) in a final volume of 20 µL, following the manufacturer's instructions and stored at –80ºC.

3.6 Real Time PCR Analysis

Primers used for qPCR (Table 1) had been previously published and were synthesized by STABVida (Portugal). The identity of the PCR products was confirmed by cloning and subsequent sequencing (STABVida, Portugal). The qPCR reactions were performed in iQ5 Real-Time PCR Detection System (Bio-Rad), using SsoFast EvaGreen Supermix (Bio-Rad Laboratories), and prepared to a final volume of 20 µl, with a final primers concentration of 300 nM, according to the manufacturer's instructions. Thermal cycling for these experiment occurred under the following conditions: initial step at 95ºC for 30 s, followed by 40 cycles of denaturation at 95ºC for 5s, plus annealing/extension (annealing temperatures in Table 1) for 10s.

Then the melting curve analysis was performed to verify the amplicon purity and size, with a dissociation protocol from 65-95 ºC followed by gel electrophoresis. Five-point standard curves constructed with 5-fold serial dilutions of pooled cDNA were used for qPCR efficiency calculation. All samples were performed in duplicated and always included a negative control to confirm the absence of contamination. To evaluate the relative transcript levels, the $2^{-\Delta\Delta CT}$ method was used with *β-actin* and *rpl27α* as best housekeeping genes, estimated by geNorm® software to provide the most reliable normalization. The PCR efficiency for target genes ranged from 85% to 110%.

Table 2. List of specific primers used for qPCR

Gene	Primer Sequence 5'-……..-3'	Annealing T.(ºC)	Accession number	Reference
Mstn	**F:** GTACGACGTGCTGGGAGACG **R:** CGTACGATTCGATTCGCTTG	60	AF258448.1	García de la serrana et al. (2014)
MyoD2	**F:** CACTACAGCGGGGATTCAGAC **R:** CGTTTGCTTCTCCTGGACTC	60	AF478568	Jiménez-Amilburu *et al.* (2013)
Mrf4	**F:** CATCCCACAGCTTTAAAGGCA **R:** GAGGACGCCGAAGATTCACT	60	JN034421	Jiménez-Amilburu *et al.* (2013)
Myogenin	**F:** CAGAGGCTGCCCAAGGTCGAG **R:** CAGGTGCTGCCCGAACTGGGCTCG	68	EF462191	Jiménez-Amilburu *et al.* (2013)
Myf5	**F:** TGTCTTATCGCCCAAAGTGTC **R:** CTACGAGAGCAGGTGGAGAACT	64	JN034420	Jiménez-Amilburu *et al.* (2013)
MyoD1	**F:** GTTTTGTTCCAGGCGGTCT **R:** GCTGGTGTCGGTGGAGAT	60	AF478569	Garcia de la serrana *et al.* (2012)
mHC	**F:** AGCAGATCAAGAGGAACAGCC **R:** GACTCAGAAGCCTGGCGATT	60	NM131404	García de la serrana et al. (2014)
mylc2	**F:** GCTGGCAATGTGGACTACAA **R:** GAGCTGCAAAGCGACAGAG	60	-	(Salmerón, 2014)
CAPN1	**F:** CCTACGAGATGAGGATGGCT **R:** AGTTGTCAAAGTCGGCGGT	58	-	Salmerón *et al.* (2013)
CAPN2	**F:** ACCCACGCTCAGACGGCAAA **R:** CGTTCCCGCTGTCATCCATCA	61	-	Salmerón *et al.* (2013)
CAPNs1a	**F:** CGCAGATACAGCGATGAAAA **R:** GTTTTGAAGGAACGGCACAT	56	-	Salmerón *et al.* (2013)
CAPNs1b	**F:** ATGGACAGCGACAGCACA **R:** AGAGGTATTTGAACTCGTGGAAG	56	-	Salmerón *et al.* (2013)
CAPN3	**F:** AGAGGGTTTCAGCCTTGAGA **R:** CGCTTTGATCTTTCTCCACA	56	-	Salmerón *et al.* (2013)
β-actin	**F:** TCCTGCGGAATCCATGAGA **R:** GACGTCGCACTTCATGATGCT	60	X89920	Salmerón *et al.* (2013)
rpl27α	**F:** AAGAGGAACACAACTCACTGCCCCA **R:** GCTTGCCTTTGCCCAGAACTTTGTAG	68	-	Salmerón *et al.* (2013)
18S	**F:** CGAGCAATAACAGGTCTGTG **R:** GGGCATGGACTTAATCAA	60	-	Castellana *et al.* (2008)

3.7 Statistics analyses

Statistic evaluation of the data was accomplished by one-way analysis of variance (ANOVA). All variables were checked for normality and homogeneity of variance, by using the Shapiro-Wilk and the Levene test, respectively. Data transformation [log(x) and arcsin(x)] was applied when homogeneity and normality of the variables were not achieved. When these assumption where still not achieved a nonparametric test (Kruskal-Wallis H-test) was performed instead.

Where significant main effects were identified by ANOVA, individual means compared using *Tukey HSD* multiple comparison test. A significance of $p<0.05$ was applied to all statistical tests. A Spearmen's rank correlation coefficient (ρ) test was applied to all variables. Correlation was considered significant at the bilateral levels of 0.05 (*) or 0.01 (**). All tests were run with SPSS statistical analysis software (SPSS ver.22.0; Chicago, USA).

The evaluation of expression stability for the three reference genes was performed using the statistical application geNorm® (http://medgen.ugent.be/).

4 Results

4.1 Growth performance and biometric measurements

No mortalities were registered during the 69 days of trial and all fish reached commercial size (>250g). There was a general trend for creatine-fed fish to be larger. However, the final body weight and length of gilthead seabream fed the control did not differ significantly from that of fish fed the three experimental diets supplemented with creatine (Table 3). Similarly, growth rate did not increased with creatine supplementation, as no significant differences in daily growth index were registered among dietary treatments (Fig. 14).

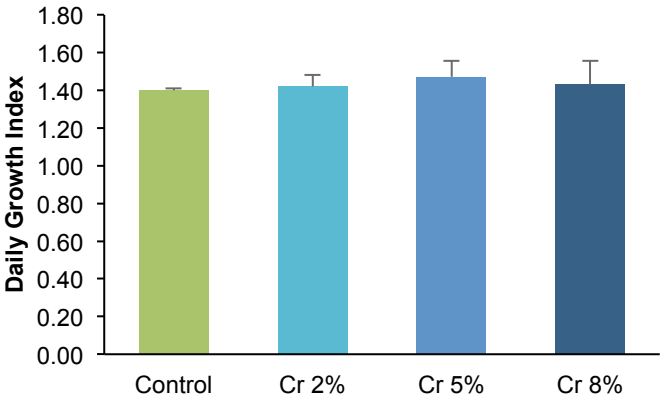

Fig. 14. DGI mean values from all treatments (mean ± SE)

4.1.1 Skeletal muscle cellularity

The dorsal muscular area of fish fed Cr 5% and Cr 8% was significantly larger than that fish fed Control and Cr 2% ($P < 0.05$) (Table 3). There was a trend for creatine-fed fish to increase the dorsal total fibres number, although no significant differences were found ($P < 0.05$) (Table 3). The mean diameter of white fibres had also a tendency to increase as the creatine supplementation increased (Table 3), but no significant difference could be observed. No significant differences diet-induced changes were observed in the distribution of skeletal white muscle fibre diameters (Fig. 15b). Muscle fibre diameter ranged from less than 20 μm to a maximum of 160 μm (Fig. 15b). The larger overall mean muscle fibre diameter observed in the group supplemented with creatine 8% seems to be mainly due to a higher relative number of large-sized fibres (≥120μm) (Fig. 15b).

Table 3. Summary of the skeletal muscle cellularity parameters

	Control	Cr 2%	Cr 5%	Cr 8%
Final Weight (g)	272.14 ± 18.92	274.98 ± 17.36	291.29 ± 23.60	288.32 ± 29.32
Length (cm)	22.75 ± 0.90	22.86 ± 0.39	22.96 ± 0.73	22.92 ± 0.45
Dorsal Muscular Area (mm²)	771.83 ± 46.99^{b}	798.44 ± 71.69^{b}	933.04 ± 22.16^{a}	899.51 ± 82.98^{a}
Fibres nº / mm²	170.47 ± 12.94	166 ± 18.80	166.55 ± 21.14	149.18 ± 12.32
Dorsal total fibre number x1000	131.40 ± 10.57	132.98 ± 22.66	150.75 ± 16.61	134.37 ± 16.55
Diameter of fibres (μm)	69.06 ± 2.38	69.59 ± 4.62	70.75 ± 2.67	73.71 ± 3.70
Fibres ≤ 20μm (%)	1.49 ± 1.12	1.99 ± 1.52	1.91 ± 1.68	1.65 ± 1.29
Fibres ≥120μm (%)	8.97 ± 1.74	9.19 ± 3.36	9.35 ± 1.81	12.19 ± 3.52

Values represent means ± standard deviation (n = 6). Means with different letters (a, b) represent significant differences between gene relative expression ($P < 0.05$)

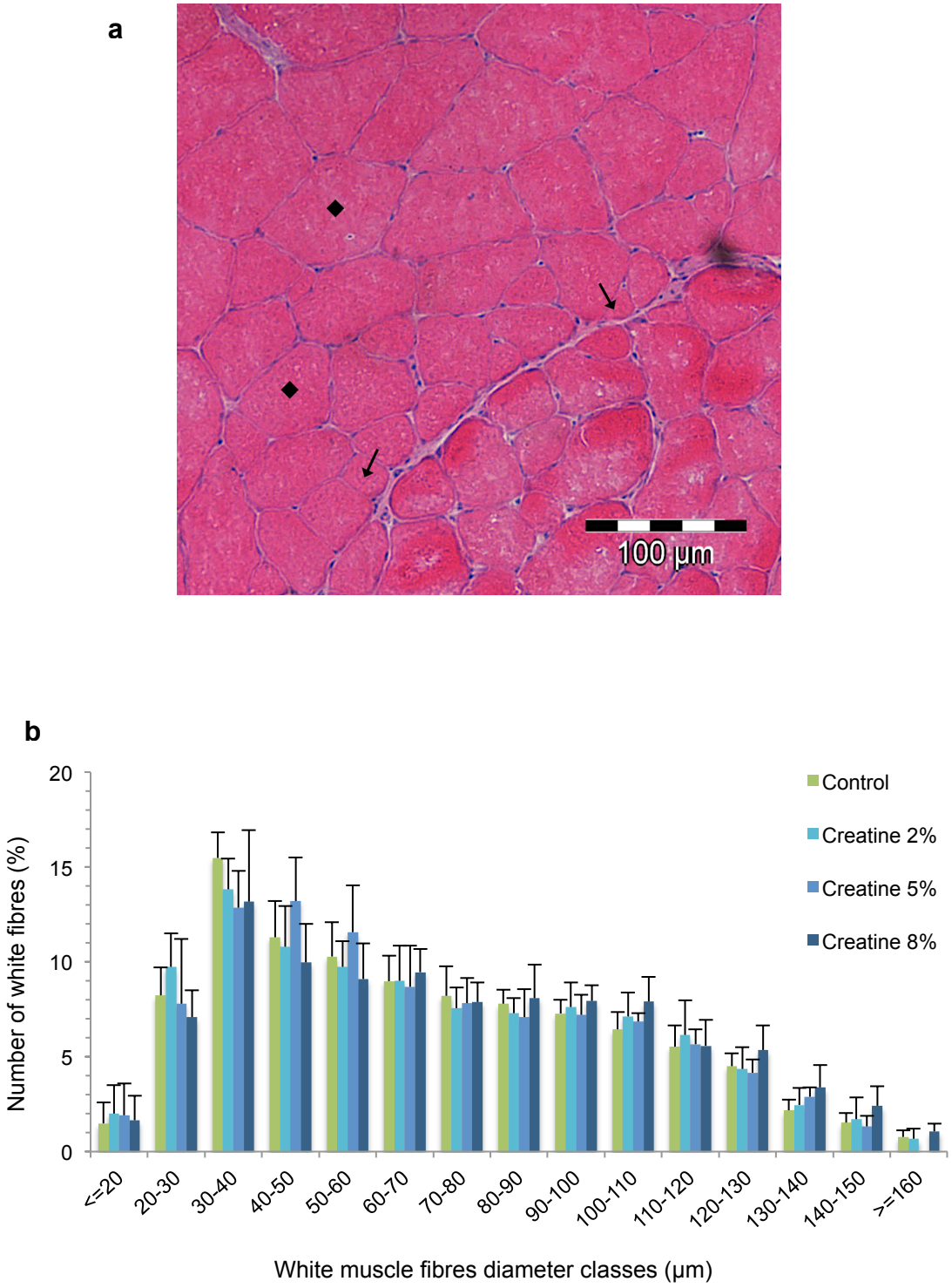

Fig. 15. Cross section of skeletal white muscle in a juvenile gilthead seabream fed 5% creatine diet, showing newly (*i.e.* small [arrow]) recruited muscle fibres between older (*i.e.* large ◆) muscle fibres (a) and (b) the distribution of white muscle fibre diameter in juvenile fed for 69 days with control and three experimental diets with different percentages of creatine supplementation (n>700).

4.2 Diet-induced plasticity of muscle-related genes

At the end of the experimental period *MyoD1* expression in white muscle, was significantly affected by (Cr 5%) creatine supplementation. Fish supplemented with 5% creatine monohydrate, the relative expression of *MyoD1* in the white muscle increased almost four times compared to those fed the control diet (P = 0.045; Fig. 16A). No significant differences could be observed among experimental diets, relative expression of other myogenic related genes (*MyoD2*, *Myf5*, *Mrf4* and *myog*) or markers of muscle structure and function (MHC) and myostatin (*Mstn*).

Creatine supplementation lead to significant differences in the expression of some genes involved in muscle proteolysis. The expression of calpain 1 (*CAPN1*) increased significantly in fish fed Cr 2% and Cr 5% (P = 0.005; Fig. 17A). But fish fed 8% creatine showed a similar *CAPN1* expression to those fed the control diet. The relative expression of calpain 3 (*CAPN3*) was highest in fish fed diets with 2% creatine supplementation but did not differ significantly from those fed the control or Cr 5%. Creatine supplementation does not seem to have significant effects on transcription levels of the other calpains analyzed (*CAPNs1a*, *CAPNs1b* and *CAPN2*).

To better understand the possible relationship between the expressions of muscle related genes and the muscle cellularity; correlations between theses parameters were performed (Table 4). Most genes were not significantly correlated with muscle phenotype. The expression was a significant and negative correlation between *myog* and DMA and a positive correlation between fibre diameter and myostatin.

Interestingly, the expression of several myogenesis-related genes was significantly correlated with genes from the calpain family. Both *MyoD* paralogues in muscle (*MyoD1* and *MyoD2*) were significantly correlated with genes from the calpain family. *MyoD1* had a positive correlation with *CAPN1* (ρ = 0.804**), as well as with *CAPNs1a* expression (ρ = 0.650*) and with *CAPN3* (ρ = 0.580*). Similarly, *MyoD2* showed a strong positive correlation with *CAPN1* (ρ = 0.727**), *CAPN3* (ρ = 0.762**), *CAPN2* (ρ = 0.594*), and *CAPNs1a* expression (ρ = 0.643*). *Myf5* was significantly correlated with *CAPN2* expression (ρ = 0.769**) (Table 4). And *Mrf4* was significantly correlated with *CAPN1* (ρ = 0.790**) (Table 4).

On the other hand only two correlations were found between gene expression and muscle growth dynamics parameters analyzed. A positive correlation was found between *Mstn* and fibre diameter (ρ = 0.664*). While a *myog* expression levels was negatively correlated with DMA (ρ = -0.622*) (Table 4), with the highest DMA observed in the group supplemented with creatine 5% (Table 3). Although not significant the relative expression of *Myog* decreases with increasing creatine supplementation (Fig. 16E).

Table 4. Correlations between gene expression in muscle and muscle growth dynamics parameters (DMA and fibre diameter)

	DMA	Fiber diameter	*CAPN1*	*CAPNs1a*	*CAPN2*	*CAPN3*
MyoD1	NS	NS	ρ = 0.804**	ρ = 0.650*	NS	ρ = 0.580*
MyoD2	NS	NS	ρ = 0.727**	ρ = 0.643**	ρ = 0.594*	ρ = 0.762**
Myf5	NS	NS	NS	NS	ρ = 0.769**	NS
Mrf4	NS	NS	ρ = 0.790**	NS	NS	NS
Mstn	NS	ρ = 0.664*	NS	NS	NS	NS
Myog	ρ = -0.622*	NS	NS	NS	NS	NS

NS: not significant. Spearmen's rank correlation coefficient (ρ) was performed. Correlation was considered significant at the bilateral levels of 0.05 (*) or 0.01 (**).

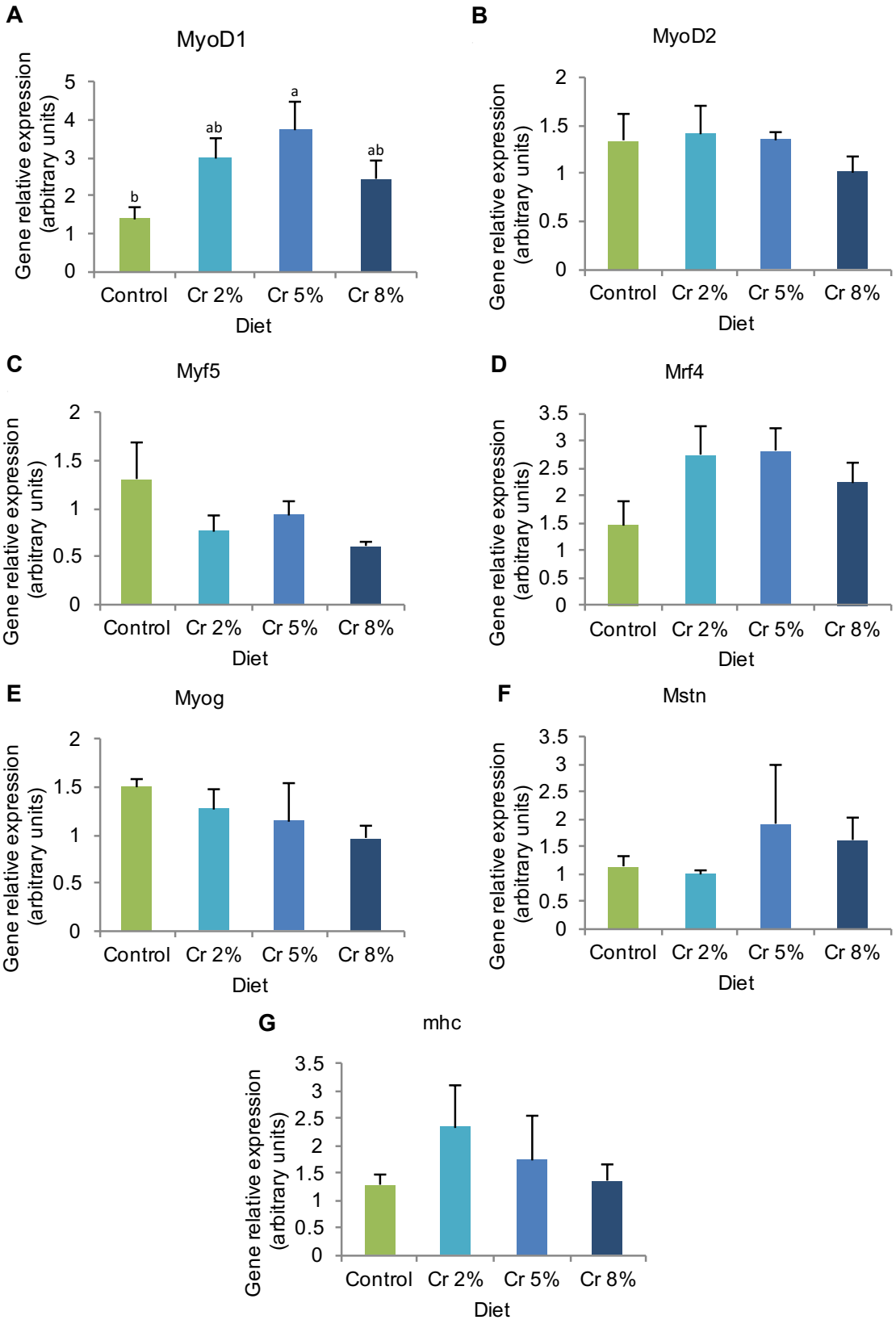

Fig. 16. Relative expression of myogenic genes and markers of muscle structure and function of gilthead seabream fed the control and the three increasing dietary creatine levels (0, 2, 5 and 8%). Different lower case letters indicate significant differences between groups. $P < 0.05$. Values presented as mean ± standard error.

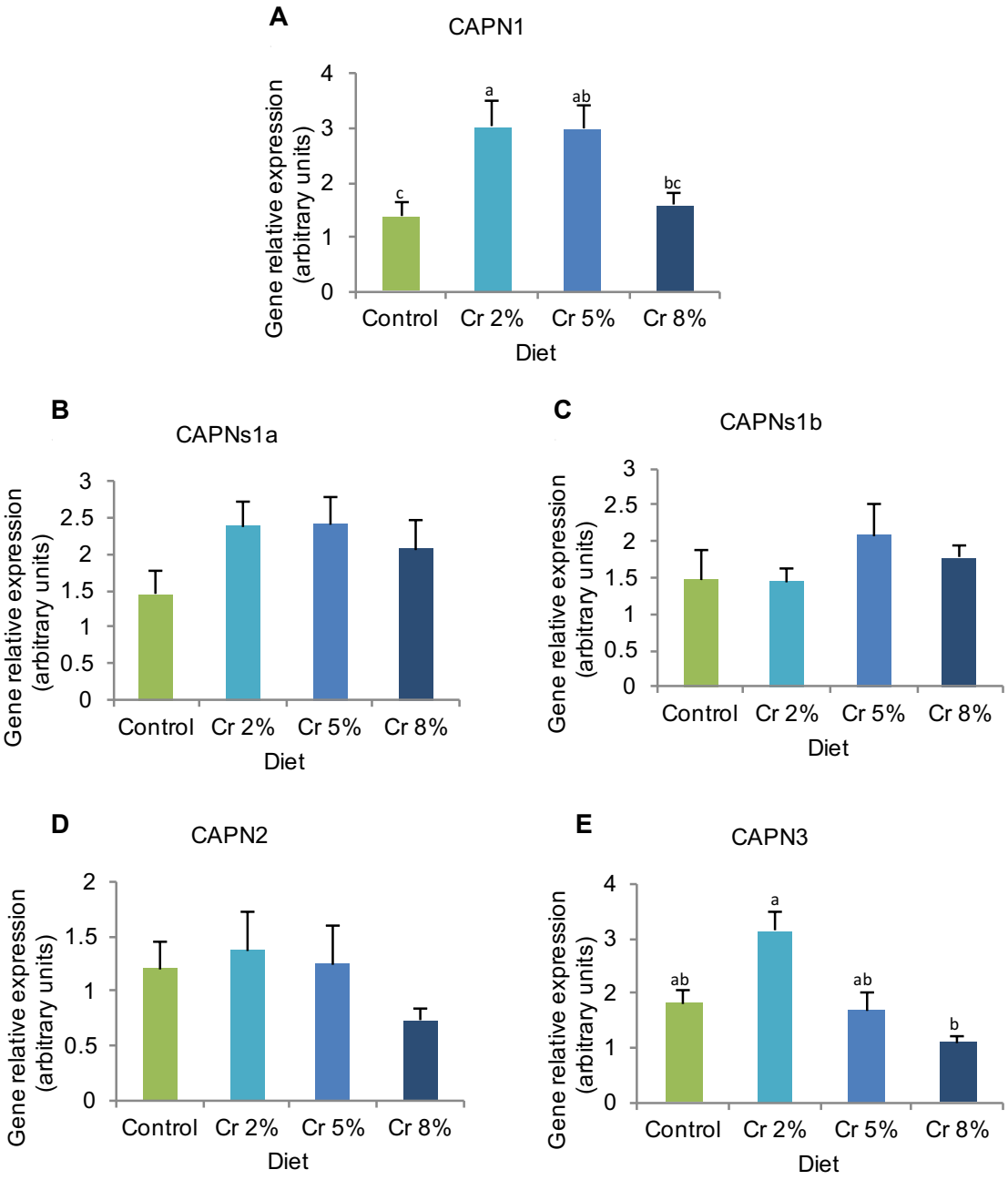

Fig. 17. Relative expression of genes involved in proteolysis in gilthead seabream fed the control and the three increasing dietary creatine levels (0, 2, 5 and 8%). Different lower case letters indicate significant differences between groups. $P < 0.05$. Values presented as mean ± standard error.

5 Discussion

Creatine supplementation has been used for many years in athletes in order to promote body mass growth and to improve their training resistance. A relatively large number of scientific studies have demonstrated increased lean body mass as a result of creatine supplementation combined with strength training (Volek *et al.*, 1997; Volek *et al.*, 1999; Branch, 2003). Ingwall *et al.* (1972) suggested that Cr may play a direct role in myosin synthesis. Yet, it is still not very clear if creatine supplementation *per se* is enough to promote such effects or if it has to be combined with training stimulus (Gualano *et al.*, 2010).

Studies concerning the effect of creatine in teleost fish are extremely scarce. A short-term dietary creatine supplementation in juvenile rainbow trout in sprint endurance did not result in a significant effect on specific growth rate (% body weight change.d^{-1}) or in muscle total creatine level (McFarlane *et al.*, 2001). Similarly, in the present study, gilthead seabream growth (final weight and length) and DGI were not significantly improved by dietary creatine supplementation (Fig. 14 and Table 3). However, a 5-8% dietary inclusion of creatine resulted in a significant increase of dorsal muscular area (Table 3). The concomitant increase in muscle fibre diameter (muscle hypertrophy) observed in gilthead seabream fed a diet within increasing dietary supplementation of creatine, even without significance might partly explain the increased dorsal muscle area observed in the creatine-rich diets (Table 3). Moreover, a trend towards increased number of large-sized fibres (≥120µm) with increased Cr supplementation was also observed (Table 3). Greater muscle fibre hypertrophy usually implies enhanced myofibrillar protein synthesis and/or reduced degradation. But, skeletal muscle cellularity revealed no significant differences in the mean fibre diameter, among treatments. In previous studies with rats dietary Cr supplementation did not significantly affected fibre hypertrophy when used alone, neither when rats where subjected to resistance training (Aguiar *et al.*, 2011).

It is known that muscle cellularity (i.e. the number, diameter and the type of fibres, and their density) is directly related with growth and is a main determinants of muscle texture both in raw and cooked fillet (Valente *et al.*, 2013). A higher number of medium sized fibres increased muscle texture measures in Atlantic salmon assessed by a sensory taste panels (“chewiness”, “firmness”, “mouth-feel” and “dryness”) and a firm texture was associated with high muscle fibre density (Johnston *et al.*, 2000). Others studies reported a positive correlation between fibre density and several textural parameters in different species (Hatae *et al.*, 1990; Periago *et al.*, 2005). In the present study, the dietary

incorporation of Cr tended to increase the large-sized fibre number resulting in a reduction of fibre density, although without a statistical significance (P>0.05). But the impact of Cr-rich diets on fillet texture standards remains to be determined. In gilthead seabream it seems that the simple dietary creatine supplementation is not enough to promote significant effects on muscle fibre cellularity and the conjugation with resistance training might be needed to further result in a stimulation growth.

Skeletal muscle growth in teleost fish is dependent on the proliferation and differentiation of MPCs (equivalent to mammalian satellite cells) that express several transcriptional activators: these control the expression of muscle-specific genes and contribute to hyperplasia and hypertrophy. Myogenic precursor activity is regulated by differential expression of myogenic regulatory factors involved in proliferation (*MyoD* family and *myf5*) and differentiation (*myog* and *Mrf4*) of satellite cells (Watabe, 2001). Previous studies have showed that gilthead seabream has two forms of *MyoD* (*MyoD*1 and *MyoD*2). *MyoD*1 is expressed in both white and red muscle and *MyoD*2 is specifically localized in white muscle (Tan & Du, 2002). *MyoD*1 relative expression significantly increased with Cr dietary supplementation, with the highest expression observed in fish supplemented with 5% creatine monohydrate (Fig. 16A). The increase in *MyoD1* expression in adult fish indicates that myoblast proliferation was occurring contributing to increased percentage of small-sized fibres (<20μm) and total number of muscle fibres. During the differentiation process of muscle growth in adult fish, new cells fuse to form additional fibres or are absorbed by existing fibres as they expand in diameter (hypertrophic growth) (Johnston *et al.*, 2003). In fact in gilthead seabream the upregulation of *MyoD* could be associated with increase in the total fibre number and an increase in muscle fibre diameter in fish fed creatine supplemented diet.

The present results suggest a fusion with myofibrils promoting muscle fibre hypertrophy, as a concomitant increase in muscle fibre diameter and a significant increase in DMA was observed with increasing dietary Cr supplementation (Table 3). Similarly, Aguiar *et al.* (2013), in an experiment of resistance training in rats found a strong correlation between the muscle fibre CSA and the expression of *MyoD*. The authors argue that this factor is more involved in the control of muscle mass than in fibre-type transitions (Aguiar *et al.*, 2013). Deldicque *et al.* (2007) identified a major signaling cascade by which Cr promotes the differentiation program of C_2C_{12} cells, via p38 MAPK and ERK1/2 pathway, which may increase the expression of transcription factors (*MyoD* and MEF2) capable of regulating the activation and differentiation of satellite cells necessary for the hypertrophic process. Previous studies in humans supplemented with creatine reported an increase in the expression of *Mrf4,* but no significant changes in myogenin expression (Hespel *et al.*, 2001). Moreover *Mrf4* and myogenin levels increased after creatine

supplementation, in conjunction with resistance training, and were strongly correlated with muscle creatine kinase mRNA expression (Willoughby & Rosene, 2003). In our study, Cr supplementation promoted a significant increase in *Mrf4* expression, but without statistical significance. (Fig. 16D and Table 3).

Myogenin and myostatin are both known to control myoblast differentiation and fusion that form myofibrils in several species (Watabe, 2001). In gilthead seabream the myogenin expression levels tended to decrease with increasing creatine supplementation, which could be reflected in a decrease in fibre differentiation (Fig. 16E). In fact a significantly negative correlation was observed between myogenin expression level and the total dorsal muscular area. Moreover myostatin was positively correlated with muscle fibre diameter (Table 4), simultaneous was also observed an increased of large-sized fibres (≥120μm) (Table 3). Contrarily data from previous works in aged mice, reported that myostatin inhibitors have significant positive effects on muscle fibre size and mass (LeBrasseur *et al.*, 2009; Arounleut *et al.*, 2013)

Calpains, are a group of non-lysossomal Ca^{2+}-dependent cysteine proteases, *CAPN1* and *CAPN2* regulate physiological processes like myoblast fusion, and *CAPN3* is known to play an important role in skeletal muscle homeostasis (Goll *et al.*, 2003). Overall in the present study calpains relative expression generaly increased with Cr supplementation in comparison with the control group (Fig. 17). Previous studies using μ-calpain (*CAPN1*) knockout mice, reported an increase in size and number of fast-twitch glycolytic muscle fibres, indicating that mice with *CAPN1* suppressed exhibit an increased capacity to accumulate and maintain protein (i.e proteins associated with muscle regeneration) in their skeletal muscle and a decrease in *MyoD* expression, suggesting less muscle regeneration (Kemp *et al.*, 2013). This study also revealed that myogenin protein abundance tends to be greater when calpain 1 was suppressed. Moreover Moyen *et al.*, (2004) revealed that overexpression of μ-calpain (*CAPN1*) decreased myogenin expression. Likewise, *CAPN1* increased expression was related with a gradual decrease in myogenin expression in *S. aurata* white skeletal muscle in response to Cr supplementation (Fig. 16E), resulting in increased DMA, and large-sized fibres (Table 3).

Calpain 1 and calpain 3 relative expression in the white skeletal muscle were affected by dietary creatine supplementation, while *CAPN2*, *CAPNs1a* and *CAPNs1b* remained unaffected. Calpain 1 and calpain 3 significantly differ between treatments, with both relative expression increasing significantly with dietary creatine supplementation of 2% (Fig. 17A and E respectively). In mammals a strong correlation has been shown between *CAPN3* expression levels and meat tenderness (shear force measurements) in cattle and sheep, but no direct evidence could link *CAPN3* levels with porcine tenderness (Parr *et al.*, 1999; Ilian *et al.*, 2001; Ono *et al.*, 2004). A study of characterization and

expression of several calpains (*CAPN1 CAPNs1a, CAPNs1b, CAPN2* and *CAPN3*) in gilthead seabream in relation with fillet firmness and tenderization, revealed that an increase in dietary carbohydrate content diminished growth but increased muscle texture parameters (maximal strength and elasticity of flesh). This study also showed a significant correlation with decreased CAPN1 and CAPNs1a expression (Salmerón et al., 2013). Indicating a potential of these genes as markers of flesh quality in this species (Salmerón *et al.*, 2013). Similarly results in muscle cellularity suggest that Cr supplementation may negatively affect fillet texture standards; with increased large-sized fibres (≥120μm) and a gradual decrease in fibre density (see Fig. 15b and Table3). Moreover it was observed that increasing supplementation lead to an increased relative expression of both *CAPN1* and *CAPNs1a*, but only *CAPN1* was significantly different (Fig. 17A and B).

Calpain 3 plays an important role in skeletal muscle homeostasis, as an endogenous regulator of calpain expression and proteolysis activity (Ono *et al.*, 2004; Stuelsatz *et al.*, 2010). Salmerón *et al.* (2013) found that the muscle-specific calpain 3 was not significantly correlated with muscle texture. Studies using C_2C_{12} cells researchers found that *CAPN3* is associated with the early phases of myogenic differentiation and that calpain 3 can affect the regulation of *MyoD*, but not *Myf5*, *Mrf4* and *myog*. Also *CAPN3* promoted a generation of a pool of reserve cells by decreasing the transcriptional activity of the *MyoD* via proteolysis (Stuelsatz *et al.*, 2010). But it is unlikely that it affects *MyoD* function within myotubes due to the high levels of *MyoD* present there. Different results were observed in the present study that evidences a strong positive correlation between calpain 3 and both *MyoD1* and *MyoD2* in the fast skeletal muscle (Table 4).

McFarlane *et al.* (2001) verified that fish muscle creatine and PCr levels are less susceptible to manipulation than human muscle stores by dietary supplementation or injection. Our results, however, suggest that creatine *per se* was able to significantly modify the expression of some genes (*MyoD1*, *CAPN1* and *CAPN3*) contributing to a significant increase in the 5% creatine fed group (Table 3). Moreover, DMA was negatively correlated with myogenin relative expression (Table 4).

The expression of the majority of the analyzed genes involved in skeletal muscle growth did not differ significantly after several weeks of dietary creatine supplementation. Additional effects of creatine might be more pronounced in conjunction with induce resistance training in teleost fish, but that remains to be further studied.

5.1 Future perspectives

Future studies should be performed to complement the information of the present work, and enlighten the effect of Cr supplementation in teleost fish: 1) evaluate the effect of creatine supplementation together with exercise/swimming stimulation, 2) evaluate supplementation in teleost's early life stages, when growth rate is higher, in comparison with juvenile stages, 3) further investigate the impact of dietary Cr on flesh additionally traits, namely, sensory and/or texture analysis

6 Conclusion

In summary, a 9-week dietary creatine supplementation trial in teleost fish, resulted in significant increases in the relative expression of certain genes related with myogenesis (*MyoD1*) and genes involved in proteolysis commonly associated with muscle texture (*CAPN1* and *CAPN3*). The group subjected to creatine 5% supplementation showed a significantly increase in dorsal muscular area. The effects on muscle growth dynamics parameters revealed as well a tendency, even though not significant, of an increase in muscular fibre diameter with increasing dietary creatine supplementation.

7 References

Aguiar AF, de Souza RW, Aguiar DH, Aguiar RC, Vechetti IJ, Jr., Dal-Pai-Silva M (2011) Creatine does not promote hypertrophy in skeletal muscle in supplemented compared with nonsupplemented rats subjected to a similar workload. *Nutrition research,* **31**, 652-657.

Aguiar AF, Vechetti-Júnior IJ, Souza ARW, Castan EP, Milanezi-Aguiar RC, Padovani CR, Carvalho RF, Silva MD (2013) Myogenin, MyoD and IGF-I regulate muscle mass but not fiber-type conversion during resistance training in rats. *International Journal of Sports Medicine,* **34**, 293-301.

Alami-Durante H, Médale F, Cluzeaud M, Kaushik SJ (2010a) Skeletal muscle growth dynamics and expression of related genes in white and red muscles of rainbow trout fed diets with graded levels of a mixture of plant protein sources as substitutes for fishmeal. *Aquaculture,* **303**, 50-58.

Alami-Durante H, Wrutniak-Cabello C, Kaushik SJ, Medale F (2010b) Skeletal muscle cellularity and expression of myogenic regulatory factors and myosin heavy chains in rainbow trout (*Oncorhynchus mykiss*): effects of changes in dietary plant protein sources and amino acid profiles. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **156**, 561-568.

Alexander RM (1969) The orientation of muscle fibres in the myomeres of fishes. *Journal of the Marine Biological Association of the United Kingdom,* **49**, 263-290.

Altringham JD, Ellerby DJ (1999) Fish swimming: patterns in muscle function. *Journal of Experimental Biology,* **202**, 3397-3403.

Andersen O, Dahle SW, van Nes S, Bardal T, Tooming-Klunderud A, Kjorsvik E, Galloway TF (2009) Differential spatio-temporal expression and functional diversification of the myogenic regulatory factors MyoD1 and MyoD2 in Atlantic halibut (*Hippoglossus hippoglossus*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **154**, 93-101.

Arounleut P, Bialek P, Liang L-F, Upadhyay S, Fulzele S, Johnson M, Elsalanty M, Isales CM, Hamrick MW (2013) A myostatin inhibitor (propeptide-Fc) increases muscle mass and muscle fiber size in aged mice but does not increase bone density or bone strength. *Experimental Gerontology,* **48**, 898-904.

Basurco B, Lovatelli A, García B (2011) Current Status of Sparidae Aquaculture. In: *Sparidae: Biology and Aquaculture of Gilthead Sea Bream and other Species, Biology and Aquaculture of Gilthead Sea Bream and other Species*. Wiley-Blackwell, pp. 1-50.

Bone Q (1979) 6 Locomotor Muscle. In: *Fish Physiology* (ed. by Hoar WS, Randall DJ). Academic Press, pp. 361-424.

Borchel A, Verleih M, Rebl A, Kuhn C, Goldammer T (2014) Creatine metabolism differs between mammals and rainbow trout (*Oncorhynchus mykiss*). *Springerplus,* **3**, 510.

Bowen SH (1987) Dietary Protein Requirements of Fishes — A Reassessment. *Canadian Journal of Fisheries and Aquatic Sciences,* **44**, 1995-2001.

Branch JD (2003) Effect of creatine supplementation on body composition and performance: a meta-analysis. *Int J Sport Nutr Exerc Metab,* **13**, 198-226.

Bryson-Richardson RJ, Currie PD (2008) The genetics of vertebrate myogenesis. *Nature Reviews Genetics,* **9**, 632-646.

Buckingham M, Vincent SD (2009) Distinct and dynamic myogenic populations in the vertebrate embryo. *Current Opinion in Genetics & Development,* **19**, 444-453.

Campbell RL, Davies PL (2012) Structure-function relationships in calpains. *Biochemical Journal,* **447**, 335-351.

Carani F, Duran B, Piedade W, Costa F, Almeida-Val V, Dal-Pai-Silva M (2014) Expression of Growth-Related Factors in Skeletal Muscle of Pirarucu (*Arapaima Gigas*) during Growth. *Journal of Aquaculture Research & Development,* **5**, 2155-9546.

Castellana B, Iliev DB, Sepulcre MP, MacKenzie S, Goetz FW, Mulero V, Planas JV (2008) Molecular characterization of interleukin-6 in the gilthead seabream (*Sparus aurata*). *Molecular Immunology,* **45**, 3363-3370.

Cheng J-H, Sun D-W, Han Z, Zeng X-A (2014) Texture and Structure Measurements and Analyses for Evaluation of Fish and Fillet Freshness Quality: A Review. *Comprehensive Reviews in Food Science and Food Safety,* **13**, 52-61.

Colloca F, Cerasi S (2015) Cultured Aquatic Species Information Programme. *Sparus aurata*. Cultured Aquatic Species Information Programme. In: *FAO Fisheries and Aquaculture Department*. FAO Fisheries and Aquaculture Department.

Company R, Astola A, Pendon C, Valdivia MM, Perez-Sanchez J (2001) Somatotropic regulation of fish growth and adiposity: growth hormone (GH) and somatolactin (SL) relationship. *Comparative Biochemistry and Physiology - Part C: Toxicology,* **130**, 435-445.

Conceição LEC, Morais S, Ivar Rn (2008) Tracer Studies in Fish Larvae. In: *Feeding and Digestive Functions in Fishes*. Science Publishers, pp. 349-392.

Danulat E, Hochachka P (1989) Creatine turnover in the starry flounder,Platichthys stellatus. *Fish Physiology and Biochemistry,* **6**, 1-9.

Daroit DJ, Brandelli A (2008) Implications of skeletal muscle creatine kinase to meat quality. *Journal of Animal and Feed Sciences,* **17**, 285-294.

De-Santis C, Jerry DR (2007) Candidate growth genes in finfish — Where should we be looking? *Aquaculture,* **272**, 22-38.

Deldicque L, Theisen D, Bertrand L, Hespel P, Hue L, Francaux M (2007) Creatine enhances differentiation of myogenic C2C12 cells by activating both p38 and Akt/PKB pathways. *Cell Physiology - American Journal of Physiology,* **293**, C1263-1271.

Dutt P, Croall D, Arthur JS, Veyra TD, Williams K, Elce J, Greer P (2006) m-Calpain is required for preimplantation embryonic development in mice. *BMC Developmental Biology,* **6**, 3.

East DB (2002) Biochemical Pathways of Creatine and Creatine Phosphate. In: *University of Tennessee Honers Thesis Projects*.

Espe M (2008) 9 - Understanding factors affecting flesh quality in farmed fish. In: *Improving Farmed Fish Quality and Safety* (ed by Lie Ø). Woodhead Publishing, pp. 241-264.

FAO (2014) FISHSTAT. In: *Global Aquaculture Production*. FAO, Rome, Italy.

Fauconneau B, Andre S, Chmaitilly J, Le Bail PY, Krieg F, Kaushik SJ (1997) Control of skeletal muscle fibres and adipose cells size in the flesh of rainbow trout. *Journal of Fish Biology,* **50**, 296-314.

Ferri P, Barbieri E, Burattini S, Guescini M, D'Emilio A, Biagiotti L, Del Grande P, De Luca A, Stocchi V, Falcieri E (2009) Expression and subcellular localization of myogenic regulatory factors during the differentiation of skeletal muscle C2C12 myoblasts. *J Cell Biochem,* **108**, 1302-1317.

Garcia de la serrana D, Estevez A, Andree K, Johnston I (2012) Fast skeletal muscle transcriptome of the Gilthead sea bream (*Sparus aurata*) determined by next generation sequencing. *BMC Genomics,* **13**, 181.

Georgiou S (2013) Expression of skeletal myosin light chain 2 in gilthead sea bream (*Sparus aurata*, L): regulation and correlation to growth markers. In: *Ph.D. Thesis*. University of Thessaly.

Goll DE, Thompson VF, Li H, Wei W, Cong J (2003) The calpain system. *Physiol Rev,* **83**, 731-801.

Greenhaff PL, Bodin K, Soderlund K (1994) Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *American Journal of ….*

Grigorakis K, Taylor KDA, Alexis MN (2003) Organoleptic and volatile aroma compounds comparison of wild and cultured gilthead sea bream (*Sparus aurata*): sensory differences and possible chemical basis. *Aquaculture,* **225**, 109-119.

Grigorakis K (2007) Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. *Aquaculture,* **272**, 55-75.

Gualano B, Acquesta F, Ugrinowitsch C, Tricoli V, Serrão J, Junior A (2010) Effects of creatine supplementation on strength and muscle hypertrophy: current concepts. *Revista Brasileira de Medicina do Esporte,* **16**, 219-223.

Hatae K, Yoshimatsu F, Matsumoto JJ (1990) Role of muscle fibers in contributing firmness of cooked fish. *Journal of food science,* **55**, 693-696.

Hespel P, Op't Eijnde B, Van Leemputte M, Urso B, Greenhaff PL, Labarque V, Dymarkowski S, Van Hecke P, Richter EA (2001) Oral creatine supplementation facilitates the rehabilitation of disuse atrophy and alters the expression of muscle myogenic factors in humans. *J Physiol,* **536**, 625-633.

Hill JA, Kiessling A, Devlin RH (2000) Coho salmon (*Oncorhynchus kisutch*) transgenic for a growth hormone gene construct exhibit increased rates of muscle hyperplasia and detectable levels of differential gene expression. *Canadian Journal of Fisheries and Aquatic Sciences,* **57**, 939-950.

Houlihan DF, Mathers EM, Foster A (1993) Biochemical correlates of growth rate in fish. In: *Fish Ecophysiology* (ed. by Rankin JC, Jensen F). Springer Netherlands, pp. 45-71.

Houlihan DF, Carter CG, McCarthy ID (1995) Chapter 8 Protein synthesis in fish. In: *Biochemistry and Molecular Biology of Fishes* (ed. by Hochachka PW, Mommsen TP). Elsevier, pp. 191-220.

Hunter A (1929) The creatine content of the muscles and some other tissues in fishes. *J Biol Chem,* **81**, 513 - 523.

Hurling R, Rodell JB, Hunt HD (1996) Fiber diameter and fish texture. *Journal of Texture Studies,* **27**, 679-685.

Huss H (1988) *Fresh fish, quality and quality changes: a training manual prepared for the FAO/DANIDA Training Programme on Fish Technology and Quality Control,* Food & Agriculture Org.

Ilian MA, Morton JD, Kent MP, Le Couteur CE, Hickford J, Cowley R, Bickerstaffe R (2001) Intermuscular variation in tenderness: association with the ubiquitous and muscle-specific calpains. *J Anim Sci,* **79**, 122-132.

Ingwall JS, Morales MF, Stockdale FE (1972) Creatine and the Control of Myosin Synthesis in Differentiating Skeletal Muscle. *Proceedings of the National Academy of Sciences of the United States of America,* **69**, 2250-2253.

Jaillon O, Aury JM, Brunet F, Petit JL, Stange-Thomann N, Mauceli E, Bouneau L, Fischer C, Ozouf-Costaz C, Bernot A, Nicaud S, Jaffe D, Fisher S, Lutfalla G, Dossat C, Segurens B, Dasilva C, Salanoubat M, Levy M, Boudet N, Castellano S, Anthouard V, Jubin C, Castelli V, Katinka M, Vacherie B, Biemont C, Skalli Z, Cattolico L, Poulain J, De Berardinis V, Cruaud C, Duprat S, Brottier P, Coutanceau JP, Gouzy J, Parra G, Lardier G, Chapple C, McKernan KJ, McEwan P, Bosak S, Kellis M, Volff JN, Guigo R, Zody MC, Mesirov J, Lindblad-Toh K, Birren B, Nusbaum C, Kahn D, Robinson-Rechavi M, Laudet V, Schachter V, Quetier F, Saurin W, Scarpelli C, Wincker P, Lander ES, Weissenbach J, Roest Crollius H (2004) Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature,* **431**, 946-957.

James BW, Goodband RD, Unruh JA, Tokach MD, Nelssen JL, Dritz SS (2002) A review of creatine supplementation and its potential to improve pork quality. *Journal of Applied Animal Research,* **21**, 1-16.

Jiménez-Amilburu V, Salmerón C, Codina M, Navarro I, Capilla E, Gutiérrez J (2013) Insulin-like growth factors effects on the expression of myogenic regulatory factors

in gilthead sea bream muscle cells. *General and Comparative Endocrinology,* **188**, 151-158.
Johnston IA, Moon T (1981) Fine structure and metabolism of multiply innervated fast muscle fibres in teleost fish. *Cell and Tissue Research,* **219**, 93-109.
Johnston IA, McLay HA (1997) Temperature and family effects on muscle cellularity at hatch and first feeding in Atlantic salmon (Salmo salar L.). *Canadian Journal of Zoology,* **75**, 64-74.
Johnston IA, Cole NJ (1998) Embryonic temperature modulates muscle growth characteristics in larval and juvenile herring. *Journal of Experimental Biology,* **201**, 623-646.
Johnston IA, Alderson R, Sandham C, Dingwall A, Mitchell D, Selkirk C, Nickell D, Baker R, Robertson B, Whyte D, Springate J (2000) Muscle fibre density in relation to the colour and texture of smoked Atlantic salmon (*Salmo salar* L.). *Aquaculture,* **189**, 335-349.
Johnston IA, Fernández DA, Calvo J, Vieira VLA, North AW, Abercromby M, Garland T (2003) Reduction in muscle fibre number during the adaptive radiation of notothenioid fishes: a phylogenetic perspective. *Journal of Experimental Biology,* **206**, 2595-2609.
Johnston IA, Abercromby M, Vieira VLA, Sigursteindóttir RJ, Kristjánsson BK, Sibthorpe D, Skúlason S (2004) Rapid evolution of muscle fibre number in post-glacial populations of Arctic charr *Salvelinus alpinus*. *Journal of Experimental Biology,* **207**, 4343-4360.
Johnston IA (2006) Environment and plasticity of myogenesis in teleost fish. *J Exp Biol,* **209**, 2249-2264.
Johnston IA, Lee H-T, Macqueen DJ, Paranthaman K, Kawashima C, Anwar A, Kinghorn JR, Dalmay T (2009) Embryonic temperature affects muscle fibre recruitment in adult zebrafish: genome-wide changes in gene and microRNA expression associated with the transition from hyperplastic to hypertrophic growth phenotypes. *Journal of Experimental Biology,* **212**, 1781-1793.
Johnston IA, Bower NI, Macqueen DJ (2011) Growth and the regulation of myotomal muscle mass in teleost fish. *J Exp Biol,* **214**, 1617-1628.
Katz SL (2002) Design of heterothermic muscle in fish. *J Exp Biol,* **205**, 2251-2266.
Kemp CM, Oliver WT, Wheeler TL, Chishti AH, Koohmaraie M (2013) The effects of Capn1 gene inactivation on skeletal muscle growth, development, and atrophy, and the compensatory role of other proteolytic systems. *American Society of Animal Science,* **91**, 3155-3167.
Kraemer WJ, Luk H-Y, Lombard JR, Dunn-Lewis C, Volek JS (2013) Chapter 39 - Physiological Basis for Creatine Supplementation in Skeletal Muscle. In: *Nutrition and Enhanced Sports Performance* (ed by Sen DBNK). Academic Press, San Diego, pp. 385-394.
Langley B, Thomas M, Bishop A, Sharma M, Gilmour S, Kambadur R (2002) Myostatin inhibits myoblast differentiation by down-regulating MyoD expression. *J Biol Chem,* **277**, 49831-49840.
LeBrasseur NK, Schelhorn TM, Bernardo BL, Cosgrove PG, Loria PM, Brown TA (2009) Myostatin inhibition enhances the effects of exercise on performance and metabolic outcomes in aged mice. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* **64**, 940-948.
Macqueen DJ, Johnston IA (2006) A novel salmonid myoD gene is distinctly regulated during development and probably arose by duplication after the genome tetraploidization. *FEBS Lett,* **580**, 4996-5002.
McFarlane WJ, Heigenhauser GJF, McDonald DG (2001) Creatine supplementation affects sprint endurance in juvenile rainbow trout. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **130**, 857-866.
Mommsen TP, Moon TW (2001) 8. Hormonal regulation of muscle growth. In: *Fish Physiology* (ed by Ian J). Academic Press, pp. 251-308.

Moyen C, Goudenege S, Poussard S, Sassi AH, Brustis J-J, Cottin P (2004) Involvement of micro-calpain (CAPN 1) in muscle cell differentiation. *The International Journal of Biochemistry & Cell Biology,* **36**, 728-743.

Ono Y, Kakinuma K, Torii F, Irie A, Nakagawa K, Labeit S, Abe K, Suzuki K, Sorimachi H (2004) Possible regulation of the conventional calpain system by skeletal muscle-specific calpain, p94/calpain 3. *J Biol Chem,* **279**, 2761-2771.

Ono Y, Sorimachi H (2012) Calpains: an elaborate proteolytic system. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics,* **1824**, 224-236.

Palstra AP, Planas JV (2012) *Swimming Physiology of Fish: Towards Using Exercise to Farm a Fit Fish in Sustainable Aquaculture,* Springer Science & Business Media.

Parr T, Sensky PL, Scothern GP, Bardsley RG, Buttery PJ, Wood JD, Warkup C (1999) Relationship between skeletal muscle-specific calpain and tenderness of conditioned porcine longissimus muscle. *J Anim Sci,* **77**, 661-668.

Pérez-Sánchez J, Calduch-Giner JA, Mingarro M, Vega-Rubín de Celis S, Gómez-Requeni P, Saera-Vila A, Astola A, Valdivia MM (2002) Overview of Fish Growth Hormone Family. New Insights in Genomic Organization and Heterogeneity of Growth Hormone Receptors. *Fish Physiology and Biochemistry,* **27**, 243-258.

Periago MJ, Ayala MD, López-Albors O, Abdel I, Martínez C, García-Alcázar A, Ros G, Gil F (2005) Muscle cellularity and flesh quality of wild and farmed sea bass, *Dicentrarchus labrax* L. *Aquaculture,* **249**, 175-188.

Pitkänen TI, Krasnov A, Teerijoki H, Xie SQ, Stickland NC, Mölsä H (2000) Growth, metabolism and tissue cellularity in fast-growing, genetically modified salmonid, arctic charr. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **126**, 120.

Poli BM, Parisi G, Scappini F, Zampacavallo G (2005) Fish welfare and quality as affected by pre-slaughter and slaughter management. *Aquaculture International,* **13**, 29-49.

Purves WK, Sadava DE, Orians GH, Craig HH (2003) *Life: The Science of Biology,* Freeman, W. H. & Company.

Randal D, Burggren W, French K (2002) *Eckert Animal Physiology: Mechanisms and Adaptations,* W. H. Freeman.

Rehbein H, Oehlenschlager J (2009) *Fishery products: Quality, safety and authenticity,* John Wiley & Sons.

Reinecke M, Bjornsson BT, Dickhoff WW, McCormick SD, Navarro I, Power DM, Gutierrez J (2005) Growth hormone and insulin-like growth factors in fish: where we are and where to go. *General and Comparative Endocrinology,* **142**, 20-24.

Rescan PY (2001) Regulation and functions of myogenic regulatory factors in lower vertebrates. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **130**, 1-12.

Rescan PY (2008) New insights into skeletal muscle development and growth in teleost fishes. *Experimental Zoology Part B: Molecular and Developmental Evolution,* **310**, 541-548.

Rowlerson A, Radaelli G, Mascarello F, Veggetti A (1997) Regeneration of skeletal muscle in two teleost fish: *Sparus aurata* and *Brachydanio rerio*. *Cell and Tissue Research,* **289**, 311-322.

Rowlerson A, Veggetti A (2001) 5. Cellular mechanisms of post-embryonic muscle growth in aquaculture species. In: *Fish Physiology* (ed by Ian J). Academic Press, pp. 103-140.

Safdar A, Yardley NJ, Snow R, Melov S, Tarnopolsky MA (2008) Global and targeted gene expression and protein content in skeletal muscle of young men following short-term creatine monohydrate supplementation. *Physiological Genomics,* **32**, 219-228.

Salem M, Yao J, Rexroad CE, Kenney PB, Semmens K, Killefer J, Nath J (2005) Characterization of calpastatin gene in fish: Its potential role in muscle growth and fillet quality. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **141**, 488-497.

Salmerón C, García de la serrana D, Jiménez-Amilburu V, Fontanillas R, Navarro I, Johnston IA, Gutiérrez J, Capilla E (2013) Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata*). *PloS one,* **8**.

Salmerón C (2014) Role of leptin and ghrelin on adipose tissue and study of muscle proteolysis in fish. In: *Departament de Fisiologia i Immunologia*. Universitat de Barcelona*,* pp. 335.

Sänger AM, Stoiber W (2001) 7. Muscle fiber diversity and plasticity. In: *Fish Physiology* (ed by Ian J). Academic Press*,* pp. 187-250.

Santinha PJM, Gomes EFS (1996) Effects of protein level of the diet on digestibility and growth of gilthead sea bream, *Sparus auratus* L. *Aquaculture Nutrition,* **2**, 81-87.

Snow RJ, Murphy RM (2001) Creatine and the creatine transporter: a review. *Mol Cell Biochem,* **224**, 169-181.

Sola L, Moretti A, Crosetti D, Karaiskou N, Magoulas A, Rossi AR, Rye M, Triantafyllidis A, Tsigenopoulos CS (2007) Genetic effects of domestication, culture and breeding of fish and shellfish, and their impacts on wild populations. Gilthead seabream - *Sparus aurata*. In: *Genetic impact of aquaculture activities on native populations. Genimpact final scientific report* (ed. by Svåsand T, Crosetti D, García-Vázquez E, Verspoor E)*,* pp. 47-54.

Steinbacher P, Haslett JR, Obermayer A, Marschallinger J, Bauer HC, Sanger AM, Stoiber W (2007) MyoD and Myogenin expression during myogenic phases in brown trout: a precocious onset of mosaic hyperplasia is a prerequisite for fast somatic growth. *Developmental Dynamics,* **236**, 1106-1114.

Stuelsatz P, Pouzoulet F, Lamarre Y, Dargelos E, Poussard S, Leibovitch S, Cottin P, Veschambre P (2010) Down-regulation of MyoD by calpain 3 promotes generation of reserve cells in C2C12 myoblasts. *J Biol Chem,* **285**, 12670-12683.

Takano J, Tomioka M, Tsubuki S, Higuchi M, Iwata N, Itohara S, Maki M, Saido TC (2005) Calpain mediates excitotoxic DNA fragmentation via mitochondrial pathways in adult brains: evidence from calpastatin mutant mice. *J Biol Chem,* **280**, 16175-16184.

Tan X, Du SJ (2002) Differential expression of two MyoD genes in fast and slow muscles of gilthead seabream (*Sparus aurata*). *Dev Genes Evol,* **212**, 207-217.

Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J, Kambadur R (2000) Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *J Biol Chem,* **275**, 40235-40243.

Turner CE, Gant N (2014) Chapter 2.2 - The Biochemistry of Creatine. In: *Magnetic Resonance Spectroscopy* (ed. by Stagg C, Rothman D). Academic Press, San Diego*,* pp. 91-103.

Valente LMP, Gomes EFS, Fauconneau B (1998) Biochemical growth characterization of fast and slow-growing rainbow trout strains: effect of cell proliferation and size. *Fish Physiology and Biochemistry,* **18**, 213-224.

Valente LMP, Rocha E, Gomes EFS, Silva MW, Oliveira MH, Monteiro RAF, Fauconneau B (1999) Growth dynamics of white and red muscle fibres in fast- and slow-growing strains of rainbow trout. *Journal of Fish Biology,* **55**, 675-691.

Valente LMP, Moutou KA, Conceição LEC, Engrola S, Fernandes JMO, Johnston IA (2013) What determines growth potential and juvenile quality of farmed fish species? *Reviews in Aquaculture,* **5**, S168-S193.

Van Leeuwen JL (1999) A mechanical analysis of myomere shape in fish. *Journal of Experimental Biology,* **202**, 3405-3414.

Venou B, Alexis MN, Fountoulaki E, Nengas I, Apostolopoulou M, Castritsi-Cathariou I (2003) Effect of extrusion of wheat and corn on gilthead sea bream (*Sparus aurata*) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities. *Aquaculture,* **225**, 207-223.

Ventura-Clapier R, De Sousa E, Veksler V (2002) Metabolic myopathy in heart failure. *News in Physiological Sciences,* **17**, 191-196.

Vergara J, Fernández-Palacios H, Robainà L, Jauncey K, Higuera M, Izquierdo M (1996a) The Effects of Varying Dietary Protein Level on the Growth, Feed Efficiency, Protein Utilization and Body Composition of Gilthead Sea Bream Fry. *Fisheries Science,* **62**, 620-623.

Vergara JM, Jauncey K (1993) Studies on the use of dietary energy by gilthead sea bream (*Sparus aurata* L.) juveniles. *Fish Nutrition in Practice. Les Colloques,* **61**, 453-458.

Vergara JM, Robainà L, Izquierdo M, Higuera MDL (1996b) Protein sparing effect of lipids in diets for fingerlings of gilthead sea bream. *Fisheries Science,* **62**, 624-628.

Vergara JM, Lopez-Calero G, Robaina L, Caballero MJ (1999) Growth, feed utilization and body lipid content of gilthead seabream (*Sparus aurata*) fed increasing lipid levels and fish meals of different quality. *Aquaculture,* **179**, 35-44.

Videler JJ (1993) *Fish swimming,* Springer Science & Business Media.

Vlachos I (2013) Consumers' perceptions of food quality products: Greece's experiences. In: *Consumer attitudes to food quality products* (ed. by Klopčič M, Kuipers A, Hocquette J-F). Wageningen Academic Publishers, pp. 247-259.

Volek J, Ballard K, Forsythe C (2008) Overview of Creatine Metabolism. In: *Essentials of Creatine in Sports and Health* (ed. by Stout J, Antonio J, Kalman D). Humana Press, pp. 1-23.

Volek JS, Kraemer WJ, Bush JA, Boetes M, Incledon T, Clark KL, Lynch JM (1997) Creatine Supplementation Enhances Muscular Performance During High-Intensity Resistance Exercise. *Journal of the American Dietetic Association,* **97**, 765-770.

Volek JS, Duncan ND, Mazzetti SA, Staron RS, Putukian M, Gomez AL, Pearson DR, Fink WJ, Kraemer WJ (1999) Performance and muscle fiber adaptations to creatine supplementation and heavy resistance training. *Medicine & Science in Sports & Exercise* **31**, 1147-1156.

Walker JB (1979) Creatine: biosynthesis, regulation, and function. *Adv Enzymol Relat Areas Mol Biol,* **50**, 177-242.

Wallimann T (2007) Introduction – Creatine: Cheap Ergogenic Supplement with Great Potential for Health and Disease. In: *Creatine and Creatine Kinase in Health and Disease* (ed. by Salomons G, Wyss M). Springer Netherlands, pp. 1-16.

Walsh FS, Celeste AJ (2005) Myostatin: a modulator of skeletal-muscle stem cells. *Biochem Soc Trans,* **33**, 1513-1517.

Wang Q, McPherron AC (2012) Myostatin inhibition induces muscle fibre hypertrophy prior to satellite cell activation. *J Physiol,* **590**, 2151-2165.

Watabe S (2001) 2. Myogenic regulatory factors. In: *Fish Physiology* (ed by Ian J). Academic Press, pp. 19-41.

Weatherley AH, Gill HS (1982) Influence of bovine growth hormone on the growth dynamics of mosaic muscle in relation to somatic growth of rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology,* **20**, 165-172.

Willoughby DS, Rosene J (2001) Effects of oral creatine and resistance training on myosin heavy chain expression. *Medicine & Science in Sports & Exercise,* **33**, 1674-1681.

Willoughby DS, Rosene JM (2003) Effects of oral creatine and resistance training on myogenic regulatory factor expression. *Medicine & Science in Sports & Exercise,* **35**, 923-929.

Wood AW, Duan C, Bern HA (2005) Insulin-like growth factor signaling in fish. *International Review of Cytology,* **243**, 215-285.

Wyss M, Kaddurah-Daouk R (2000) Creatine and creatinine metabolism. *Physiol Rev,* **80**, 1107-1213.

Zhivotovsky B, Orrenius S (2011) Calcium and cell death mechanisms: A perspective from the cell death community. *Cell Calcium,* **50**, 211-221.

Zimmerman UJ, Boring L, Pak JH, Mukerjee N, Wang KK (2000) The calpain small subunit gene is essential: its inactivation results in embryonic lethality. *IUBMB Life,* **50**, 63-68.